Parallelism and Epistasis in the de novo Evolution of Cooperation between Two Species

Parallelism and Epistasis in the *de novo* Evolution of Cooperation Between Two Species

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Sarah Michael Douglas

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Abstract

Resolving the genetic and mechanistic bases of complex biological behaviors remains a central challenge in the post-genomic era. Among these is the emergence of interspecies cooperation, a feature common across levels of biological organization. Of the numerous examples afforded by nature, microbes arguably provide the greatest ability to connect underlying genotypes to cooperative phenotypes.

Using an engineered bacterial consortium, we repeatedly evolved cooperation and tested how interspecies dynamics impact the predictability of evolution. Eight *Salmonella enterica* serovar Typhimurium strains evolved methionine excretion sufficient to support growth of an *Escherichia coli* methionine auxotroph, from whom they required excreted growth substrates. Non-synonymous mutations in *metA*, encoding homoserine trans-succinylase, were detected in each evolved *S. enterica* methionine cooperator, and were shown to be necessary for cooperative consortia growth. Despite this genetic parallelism, these *metA* alleles gave rise to a wide range of phenotypic diversity in terms of individual versus group benefit. The cooperators with the highest methionine excretion permitted nearly two-fold faster consortia growth and supported the highest fraction of *E. coli*, yet interestingly also had the slowest individual growth rates compared to less cooperative strains.

The two-step selective protocol used to evolve these cooperators, however, raised questions about historical contingency, which is believed to play a large role in shaping evolution. Without initial selection for resistance to end-product transcriptional inhibition, *S. enterica* struggled to evolve cooperation. Selection for resistance to a toxic methionine analog, ethionine, enabled more efficient evolution of cooperation by *S. enterica* in a process that required two adaptive mutations. When bacteria must overcome multiple levels of metabolic repression to excrete costly compounds, gene interactions like epistasis may limit adaptive strategies. In this consortium, epistasis between *metJ* and *metA* adaptive mutations suggests microbes undergoing *de novo* evolution of cooperation face similar challenges.

Knowing how previous selective pressures and interspecies dynamics impact adaptive variation at the genetic, phenotypic, and ecological levels will better constrain our ability to predict complex microbial community behavior from the genotypes or phenotypes of the strains within them.

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Acknowledgements

I have run twelve marathons in my life, and graduate school has been – by far – the most challenging endurance test. If I have finished this race hale and with my joy in science intact, it is only due to that wealth of mentors and support I have found at during my graduate career.

First and foremost I would like to thank my advisor Chris Marx for helping guide my development as scientist. Chris gave me the freedom, help, and tools to develop my own ideas, dig my own holes, and find my own way out. In addition to his invaluable advice and mentorship, he gifted me with a deep appreciation of microbiology in all the forms, but especially beer.

William Harcombe acted as a second mentor to me during his time as a post-doc in the Marx Lab. My debt of gratitude to Will cannot be overstated, and not just because he taught me sterile technique. Beyond his creation of the fabulous *S. enterica – E. coli* consortium that gifted me with such rich research opportunities, Will taught me how to think like an evolutionary biologist. His creativity and clarity of thought have helped me arrive at some of my most exciting revelations about microbial evolution.

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I would also like to thank Andrew Murray, Karine Gibbs, and Stephen Lory for their thoughtful and helpful guidance during my graduate career. They've provided

invaluable and necessary outside perspective throughout my graduate work, and suggested several of the experiments that help make this dissertation a more complete body of work.

It takes a village to raise a graduate student, and the Marx Lab was my village. All current members of the lab have reviewed parts of this dissertation and offered excellent advice. Lab managers Maryska Kaczmarek and Paige Swanson kept the lab afloat and running with an enviable efficiency. Nigel Delaney, Nick Leiby, and Jose Rojas Echenique helped me learn the essentials of programing. Josh Michener optimized the qPCR I used in my 11^{th} hour experiments, and was an invaluable source of technical expertise during that dark hour. The other members of the lab –past and present – have all been a source of technical and moral support, ready to supply advice or a drink as needed.

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"Even his griefs are a joy long after

to one that remembers all that he wrought and endured."

— Homer, *The Odyssey*

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CHAPTER ONE

Introduction

> *"Evolutionary speculation constitutes a kind of metascience, which has the same intellectual fascination for some biologists that metaphysical speculation possessed for some mediaeval scholastics. It can be considered a relatively harmless habit, like eating peanuts, unless it assumes the form of an obsession; then it becomes a vice."* – Roger Stanier on microbial evolution, 1970[1]

Before the advent of affordable nucleic acid sequencing, the study of dynamic microbial systems was problematic, mired in difficulties determining phylogeny[1]. Now, in the current genomic era, sequencing has become a first-wave tool to determine and summarize the character of natural biotic systems[2–4]. Using this sequencing ability, microbiology's traditional focus on the study of species' behavior in monoculture has begun to shift to include more analysis of complex, multi-species systems and their relevance in industrial processes[5], human health[3,4], and waste remediation[6,7]. As sequencing costs continue to plummet, the utility of this genomic "roll-call" is increasingly limited only by our understanding of how identified community components interact and change over time. For example, metagenomics can now identify species composition of these communities, and metabolic modeling can accurately predict phenotypes of individual species (reviewed in [8]). In addition, cellular optimization models have been used to accurately forecast how individual species respond to selective pressures[9]. Given the components of a system, can we predict the behavior of the assemblage over time?

Evolution is an inescapable force affecting dynamics of microbial communities. If evolution of such communities is to become a predictive science, we need a better understanding of the types of mutations that arise from adaptation of a population, how those mutations impact organismal phenotype, and how genetic changes in one species

influence community properties. The social impact of neighboring microbes can create differing selective pressures as strong as abiotic ones, and contribute to a greater diversity of adaptations[10]. In addition, in multi-species microbial communities, species interactions can further complicate and limit our attempts to model community behavior[8,11]. It is becoming clear that behavior of a species in monoculture can be a poor predictor of its role in a community context. For example, experimental evolution of five microbial species displayed divergent evolutionary strategies when grown separately or combined in a new environment[12]. Species interactions in polyculture, arising from cross-feeding of metabolic waste productions, led to growth rates and resource utilization in community that differed significantly from those observed in monoculture growth.

Cross-feeding, or the dependence of one species' growth on substrates provided by another, is one of a growing number of social behaviors only previously associated with macroscopic organisms that are being observed more and more at the microscopic level[13]. Such social behaviors can help or hinder the growth of neighboring cells[14,15]. Antagonistic relationships between microbes include competition or predation. In addition, a variety of symbioses have been observed in natural communities, ranging from parasitism to commensalism to mutualism. One example of mutualism is the sharing of cellular products made by individual amongst the surrounding community as "public goods"[14]. Where this type of cooperation imposes a burden on individual cells, such as the production of small molecules and nutrients, there becomes a challenge to explain how these costly adaptations spread in a population if they increase the fitness of unrelated individuals.

There's been much recent interest in examining how evolution impacts ecology. But few studies have focused on how those dynamics affect adaption to new environments[16]. And even fewer studies have explored the dynamics that arise when a multi-species community co-evolves to a novel environment[12]. Experimental evolution of simple bacterial systems can allow for a more nuanced study of both the initial adaptations that give rise to community-level social behavior, and the ecological consequences of these adaptations. In one rare empirical example of evolved mutualism, a sulfate-reducing bacterial species was paired with a methanogen species to form a synthetic obligate mutualism in 24 replicate populations[17]. While most communities eventually improved growth rate and yield over 300 community doublings, initial evolution was marked by instability and the role of the methanogen partner in growth rate differed greatly between communities. By repeatedly evolving such inter-species interactions, we can probe the diversity of molecular solutions available to the microbes, and characterize the phenotypic and ecological consequences of such mutations.

The consortium used here to examine the evolution of cooperation is comprised of two-members - *Escherichia coli* K12 and *Salmonella enterica* serovar Typhimurium[18]. Both members have a reciprocal requirement of the other for growth. The cooperative adaptation is methionine excretion by *S. enterica* in sufficient quantities to support growth of an *E. coli* methionine auxotroph (*E. coli* Δ*metB*), which, in turn, supplies *S. enterica* with growth substrates.

Evolution of cooperation in this system is a two-step process. Previous studies of the methionine biosynthetic pathway in *S. enterica* evolved methionine overproduction in a single-step by selecting for resistance to toxic methionine analogs, like ethionine[19,20]. In

this consortium, resistance to ethionine did not result in methionine excretion by *S. enterica* sufficient to support *E. coli* Δ*metB*. A second selection step, where the ethionine-resistant *S. enterica* was co-cultured with the *E. coli* partner, did result in evolving an *S. enterica* cooperator. This first *S. enterica* evolved in the consortium exhibited slow individual growth, showing this cooperation to be costly. As such, its evolution was only observed on agarose plates, rather than in liquid media. Genotypes that produce public goods must disproportionately benefit from their cooperation, a dynamic that can occur in a spatially structured environment, which correlates genotypes to their own local environment.

The ability to evolve a mutualism *de novo* provides a rare opportunity to observe how the added factors of evolution and interspecies interactions impact population dynamics in a new environment. In the work presented here, I sought to uncover how both factors affect the predictability of community behavior. For example, observation of adaptations that repeatedly target the same gene or phenotype in independent populations, termed "parallel evolution," can lead to more accurate predictions about future evolution of similar communities. Parallel evolution can occur when closely-related lineages evolve the same phenotype, and is one sign of adaptation by natural selection. In the experimental evolution of microbes, where replicate populations are introduced into a new environment and allowed to accrue adaptations over many generations, parallelism is often observed at the phenotypic[21,22] and genetic[23–26] levels in monoculture. Mutation bias, clonal interference, or epistatic interactions may limit or bias evolutionary trajectories and give rise to this parallelism[27,28]. In addition, the existence of one superior solution in large populations that efficiently sort all possible adaptations may result in

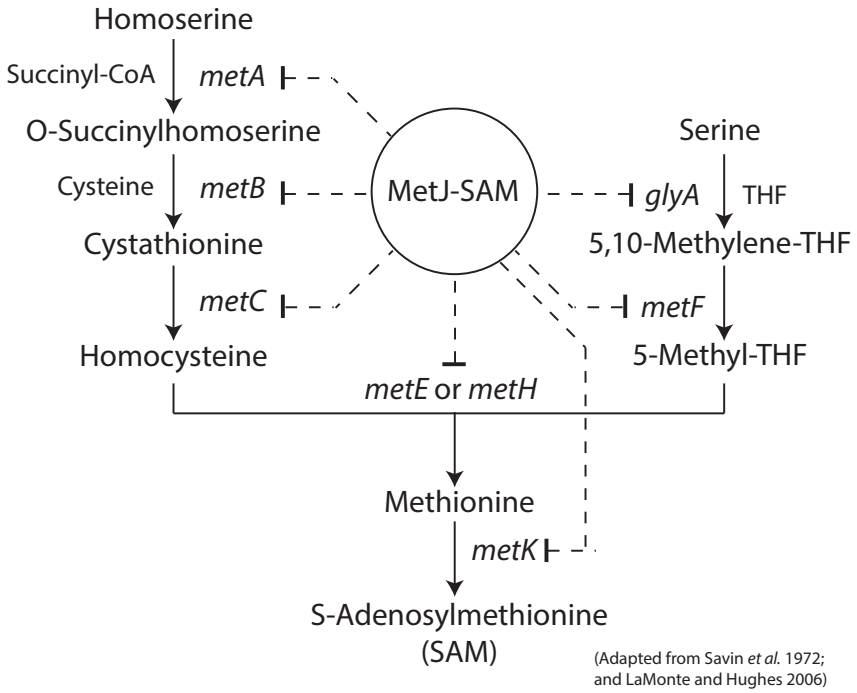

Figure 1.1. **The methionine biosynthetic pathway in *S. enterica* is transcriptionally repressed by MetJ-SAM**

similar repeatability. Consistently observed mechanisms of adaptation supports the idea that genetic evolution in these populations may be somewhat predictable, and can be used to model future responses to similar environments.

In our consortium, we observed a remarkable degree of genetic parallelism at each step of selection for cooperation. In previous studies, resistance to methionine analogs has come from diverse mutational targets within the methionine biosynthetic pathway, including *metA*[29], *metK*[19,30], and *metJ*[19] (Figure 1.1). The ethionine-resistant backgrounds that gave rise to cooperators, however, all featured mutations in *metJ*, a transcriptional regulator of the methionine pathway (Chapter 3). From a metabolic engineering viewpoint, this result makes sense, since regulatory mutations often precede structural changes during enzyme evolution within novel environments[31]. The second

step of our pathway, the co-culturing of ethionine-resistant *S. enterica* with *E. coli* Δ*metB*, gave rise to eight producers, all of which have a mutation in *metA* (Chapter 2). This parallelism in the second step may arise from the type of cooperation under selection. Within a community context, conditions that select for cooperation can directly act on metabolic traits, which can be determined by a single gene. Thus metabolic changes can be linked to large-scale ecological changes when a metabolic compound is the currency of exchange in a cross-feeding dependency.

This genetic parallelism, though, gave rise to a spectrum of cooperative strategies that reveal a consistent trade-off between adaptation to abiotic and biotic factors. Such trade-offs, once defined, can be utilized to model future community response to novel environments[16]. A project that initially began as a basic attempt to characterize the molecular mechanism of cooperative evolution can now help elucidate community behaviors in two-species system that incorporates the dynamics of social interaction and evolution.

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CHAPTER TWO

Parallel Mutations in a Single Gene Result in a Range of Cooperative Strategies in a Bacterial Consortium

Sarah M. Douglas[1,2], Lon M. Chubiz[2], William R. Harcombe[2,a], Christopher J. Marx[2,3,4,5*]

[1]Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, United States of America

[2]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts, United States of America

[3]Faculty of Arts and Sciences Center for Systems Biology, Harvard University, Cambridge, Massachusetts, United States of America

[4]Department of Biological Sciences, University of Idaho, Moscow, Idaho, United States of America

[5]Institute for Bioinformatics and Evolutionary Studies, University of Idaho, Moscow, Idaho, United States of America

[a]Current address: Department of Ecology, Evolution and Behavior, University of Minnesota, St. Paul, Minnesota, United States of America

*Email: cmarx@uidaho.edu

Abstract

Multi-species microbial communities play a critical and often underappreciated role in human health, industry, and waste remediation. Species interactions like cooperation complicate attempts to predict how these communities will adapt to environmental challenges. Using an engineered bacterial consortium, we repeatedly evolved cooperation and tested how interspecies dynamics impact the predictability of evolution. Eight *Salmonella enterica* serovar Typhimurium strains evolved methionine excretion sufficient to support growth of an *Escherichia coli* methionine auxotroph, from whom they required excreted growth substrates. Non-synonymous mutations in *metA*, encoding homoserine trans-succinylase (HTS), were detected in each evolved *S. enterica* methionine cooperator and were shown to be necessary for cooperative consortia growth. Despite this genetic parallelism, these *metA* alleles gave rise to a wide range of phenotypic diversity in terms of individual versus group benefit. The cooperators with the highest methionine excretion permitted nearly two-fold faster consortia growth and supported the highest fraction of *E. coli*, yet interestingly also had the slowest individual growth rates compared to less cooperative strains. Knowing how interspecies dynamics impact adaptive variation at the genetic, phenotypic, and ecological levels will better constrain our ability to predict complex microbial community behavior from the genotypes or phenotypes of the strains within them.

Introduction

There is long-standing debate over whether evolutionary outcomes are repeatable. Gould famously argued that the tape of life could not be replayed[1]. In contrast, in the experimental evolution of replicate microbial populations parallelism is often observed at the phenotypic[2,3] and genetic[4–7] levels. However, the extent to which these monoculture results will extend to more dynamic multi-species assemblages remains unclear. Furthermore, species interactions may affect how variance in genetic change relates to variance at the phenotypic and ultimately community scale.

Adaptations that arise in monoculture can be a poor predictor of evolution within a community context. Multi-species microbial communities critical to human health[8,9], industrial production[10], and waste remediation[11,12] are governed by complex social dynamics. Predicting how these communities might respond to environmental changes requires an understanding of how species interactions impact the evolution of new traits. Interspecies interactions, like competition or cooperation, complicate environmental selective pressures and decrease the predictive power of monoculture behavior[13,14]. For example, a cooperative species that excretes a beneficial cellular product, or "public good," may alter the environment and thus alter the selective pressure on other species[15]. When this cooperation is costly to individual growth, but rewarded by cooperative behavior from another species, a complex equilibrium between adaptation to the environment and adaptation to the other species complicates the predictability of evolutionary trajectories.

Evolving costly cooperation between species provides an opportunity to test how the trade-offs between adaptation to biotic versus abiotic pressures affect the

predictability of evolution. Using a synthetic bacterial system that allows *de novo* evolution of cooperation[16], we repeatedly evolved the cross-feeding of costly, mutually-dependent metabolites. This synthetic community, or consortium, is comprised of two-members: an *Escherichia coli* methionine auxotroph (Δ*metB*) and *Salmonella enterica* serovar Typhimurium. Both members have a reciprocal requirement of the other for combined growth in lactose minimal media (Figure 2.1). Wild-type *E. coli* can excrete usable carbon for *S. enterica,* which cannot metabolize lactose. *S. enterica* must, in turn, evolve sufficient methionine excretion to support growth of the *E. coli* methionine auxotroph. In the initial instance of this evolution of cooperation[16], methionine excretion by *S. enterica* was found to be individually costly, substantially decreasing the cooperator's own growth rate compared to its ancestor[16].

We repeatedly evolved costly methionine excretion using different, closely-related *S. enterica* backgrounds to examine the repeatability of evolution at the genetic, phenotypic, and ecological scales in a multi-species consortium. We examined the underlying molecular changes in our *S. enterica* mutants and found that all evolved strains featured mutations in the *metA* gene, which encodes the first step of methionine biosynthesis, homoserine trans-succinylase (HTS)[17]. In contrast to the genetic parallelism, mutations within *metA* gave rise to a range of methionine excretion and, thus, cooperative phenotypes. Thus, despite that only a single locus appears to be the main adaptive target to permit cooperation in a complex, two-species consortium, the resulting alleles can differ dramatically in how far the cell becomes committed to cooperation versus individual growth.

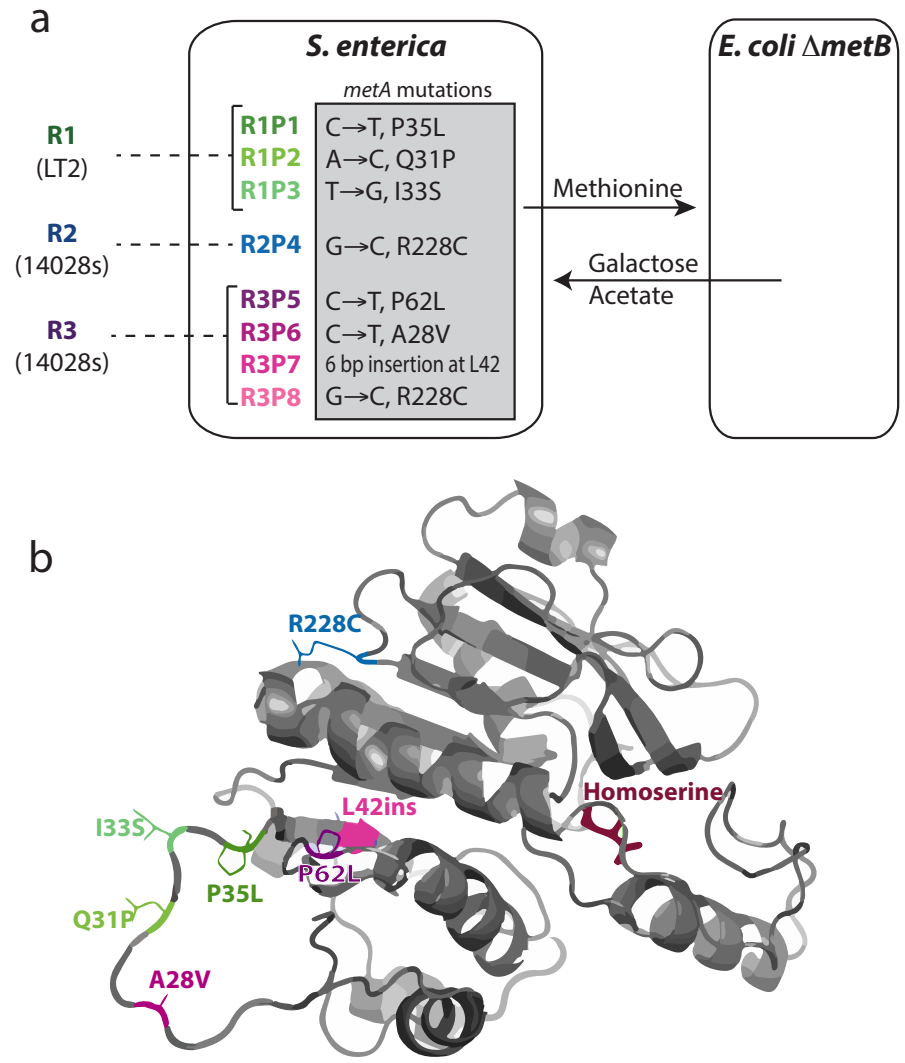

Figure 2.1. S. enterica* cooperators evolved from ethionine resistant ancestors feature mutations in *metA. a) *S. enterica* ethionine resistant strains (R strains) were co-cultured with *E. coli* methionine auxotrophs on lactose minimal media. Adaptive methionine excretion by evolved cooperators enabled growth of *E. coli* Δ*metB*, which in turn excretes usable carbon for *S. enterica*. Non-synonymous substitutions in the *metA* gene, encoding homoserine trans-succinylase (HTS) are listed next to producer strain name. b) A homology model of residues 2-297 of *S. enterica* HTS was created using *Bacillus cereus* HTS (PDB 2h2wA; 49.66% sequence identity). Each evolved cooperator features a mutation at one highlighted residue. The active site is shown with its cognate substrate homoserine.

Results

Replicate *S. enterica* cooperators were evolved from multiple starting genotypes

To explore the genetic and physiological range of adaptations underlying the rise of cooperation in our consortium, we repeatedly evolved methionine-producing *S. enterica* strains by selecting for their ability to support growth of their auxotrophic *E. coli* partner (Figure 2.1a). A classic route to generating methionine overproduction in *S. enterica* has been the selection of resistance to ethionine, a toxic methionine analog [18,19]. Methionine production in *S. enterica* is tightly regulated at the level of transcription and translation via end-product inhibition [18,20], and ethionine represses methionine biosynthesis via the same mechanism. A previously characterized Eth^R strain[16] from *S. enterica* LT2 (hereafter "R1") did not excrete detectable levels of methionine, but became the ancestor for an evolved cooperator. We selected two new Eth^R backgrounds, "R2" and "R3," from *S. enterica* 14028s. These three R strains were the ancestors for subsequent evolution of cooperation, and represent genetically distinct backgrounds: R1 is derived from the LT2 strain, while R2 and R3 were derived from 14028s. All three possess causative Eth^R mutations in *metJ* (Chapter 3). Despite their slightly differing genetic backgrounds (genomic sequences differ by 2%[21]), these R strain ancestors arise from the same *S. enterica* serovar, and thus any similarities in evolved traits would constitute parallelism.

We evolved these new *S. enterica* cooperators by plating each R ancestor with *E. coli* Δ*metB* on lactose agarose plates, and screening for lactose utilization by the *E. coli*. By repeatedly replaying the evolution of methionine excretion from each R ancestor, we obtained seven new cooperators that could support community growth (Figure 2.1a).

Genetic parallelism in cooperators evolved from different starting genotypes

Whole-genome sequencing of two of these *S. enterica* cooperators (Figure 2.1a: R1P1 and R2P4) and targeted sequencing in the other six methionine-producing lineages revealed that all evolved strains feature mutations in a single gene, *metA*, which encodes homoserine trans-succinylase (HTS) (Figure 2.1a). All cooperators feature independent point-mutations or an insertion in the coding region of HTS (termed P1 to P8), and all but two substitutions fall within the first 62 amino acids of HTS; intriguingly, none appear within the active site (Figure 2.1b). Each evolved *metA* allele is unique, with the exception of R228C in both $metA^{P4}$ and $metA^{P8}$. In all cases, sequencing *metA* in the ancestral R strains showed the original $metA^{WT}$ allele, indicating that each *metA* mutation immediately preceded the emergence of the cooperative phenotype.

***metA* mutations are necessary for cooperative phenotype**

To test the necessity of the *metA* mutations for cooperation, the ancestral $metA^{WT}$ allele was substituted into each cooperator strain, and cooperative behavior was then measured in co-culture (consortia) with *E. coli* Δ*metB*. Evolved *S. enterica* strains with the $metA^{WT}$ allele failed to recapitulate cooperation-dependent growth when grown in liquid consortia with *E. coli* Δ*metB*. The evolved *metA* alleles were not sufficient for cooperation in the wild-type *S. enterica*, as they could not restore cooperation when placed alone into wild-type LT2 background (Supplemental Figure 1). In contrast, direct substitution of any of the evolved *metA* alleles into their ancestral R background restored consortia growth rates to the levels seen from the original evolved producers (Figure 2.2).

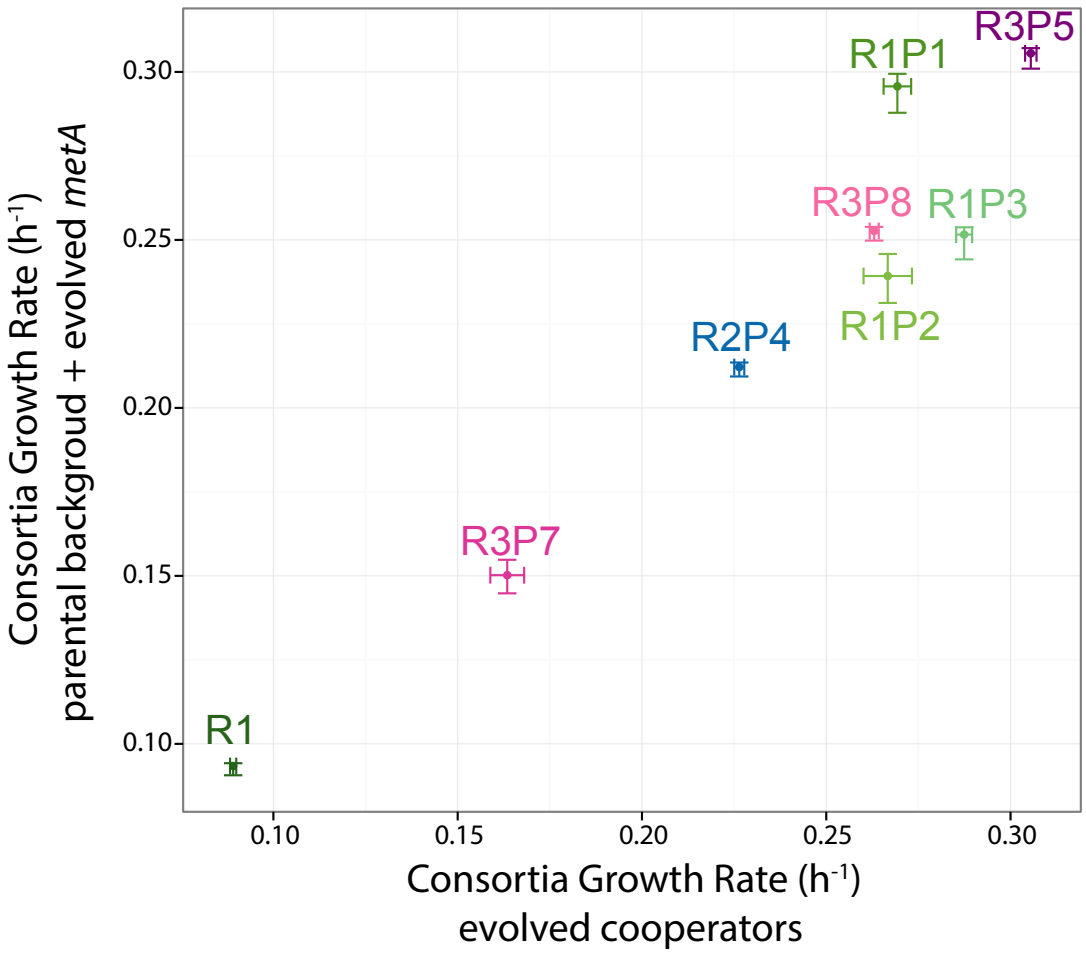

Figure 2.2. **Substituting evolved *metA* alleles into R strains recapitulates cooperative phenotype.** Consortia growth rates of R strains engineered to excrete methionine via insertion of evolved *metA* alleles correlate to growth rates of corresponding evolved strains ($R^2 = 0.930$). Error bars indicate the standard error of three biological replicates.

We attempted to measure the concentration of *metA*'s protein, HTS, by creating translational fusions to epitope-tags in the chromosomal copy of *metA*. Unexpectedly, any of the tagged ancestral HTS alleles in the R1 background directly restored consortia growth, suggesting that all of these fusions may have stabilized HTS sufficiently for methionine overproduction (Supplemental Figure 2). As discussed below, these data are

consistent with the idea that any perturbation that stabilizes HTS, an inherently unstable protein, can result in a cooperative phenotype.

Methionine excretion by evolved cooperators is highly variable

To quantify the variability of physiological consequences arising from parallel *metA* mutations in *S. enterica* evolved strains, excretion methionine was measured via gas chromatography/mass spectroscopy in conditioned media collected from *S. enterica* at mid-log phase from ancestral and evolved strains (Figure 2.3). While any metabolites downstream from the *metB*-encoded cystathione-γ-synthase enzyme in the methionine pathway would have enabled *E. coli* Δ*metB* growth, the metabolite detected in conditioned media of all evolved cooperators was methionine, the most "costly" metabolite for *S. enterica* to produce in terms of utilized cellular resources. No significant methionine concentration (>250 nM) was detectable in conditioned media from the ancestral R strains, but cooperators exhibited levels of production ranging from 0.272 to 268.2 μM in cultures normalized to an OD_{600} of 1.0. This 1000-fold range of methionine excretion represents a surprising increase in phenotypic variance among genetically-similar isolates.

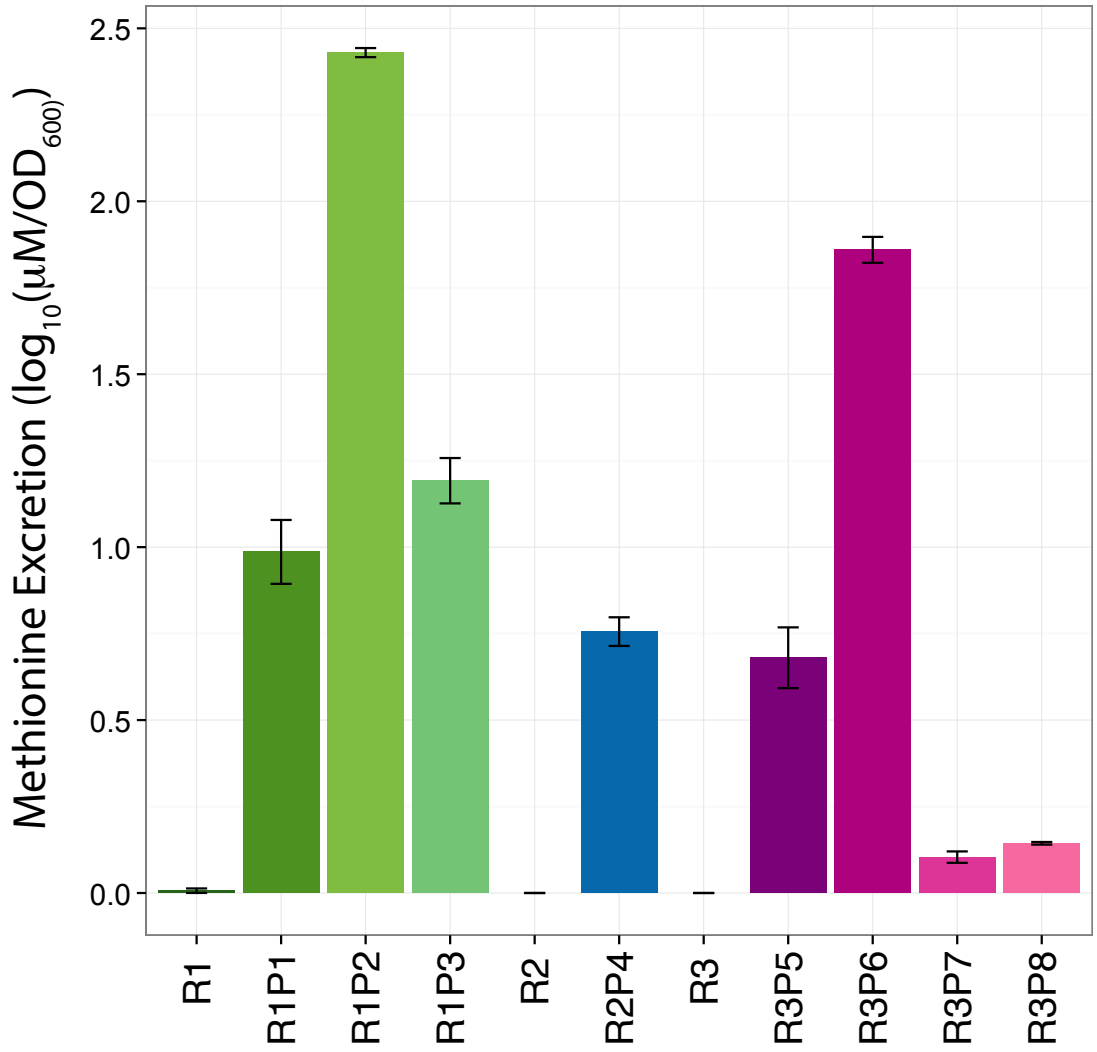

Figure 2.3. **Methionine excretion by *S. enterica* producers.** GC-MS quantification of methionine in conditioned media of ancestral and evolved *S. enterica* strains are given as log(μM) methionine per unit OD_{600}. Each data point represents three biological replicates.

Methionine excretion correlates with group performance

In order to study the ecological consequences of the highly varied methionine excretion levels, consortia growth rates for ancestral and evolved *S. enterica* strains co-cultured with *E. coli* Δ*metB* were measured in lactose minimal media. Consortia containing R

strains show slow but measurable growth (0.038-0.054 per hour), suggesting either basal *S. enterica* cell death or methionine leakage by the ancestral backgrounds is sufficient to maintain some *E. coli* growth. Consortia growth rates when using evolved strains were exponential, and varied from 0.209 to 0.362 per hour (Figure 2.4a). These growth rates were positively correlated ($R^2 = 0.773$) in a log-linear fashion with the cooperators' methionine excretion rates previously measured (Figure 2.4b).

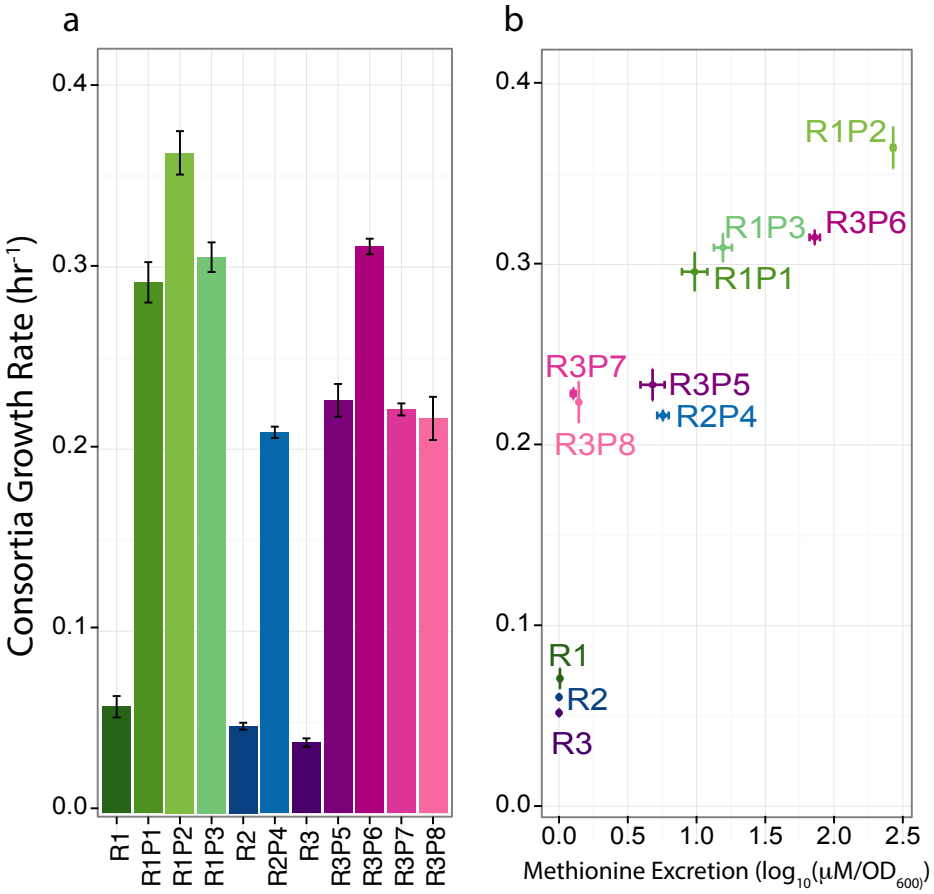

Figure 2.4. **Increased consortia growth rate for evolved strains reflects increased *S. enterica* methionine excretion.** a) Growth rate of cooperative *S. enterica* co-cultured with *E. coli* Δ*metB* in lactose minimal media. b) Consortia growth rates are positively correlated ($R^2 = 0.773$) with methionine excretion by evolved *S. enterica* cooperators. Error bars indicate standard error of three biological replicates.

Cooperation is costly to individual growth

To assess the extent to which trade-offs exist between adaptations to abiotic and biotic pressures, a trade-off that was observed in the first *S. enterica* cooperator'[16], individual growth rates of all *S. enterica* strains were measured in galactose minimal media (Figure 2.5a). Galactose is excreted by the *E. coli* Δ*metB* partner while growing in lactose minimal medium supplemented with methionine (Harcombe and Marx, unpublished). Following the effects within each lineage, the R1 strain displays the slowest initial

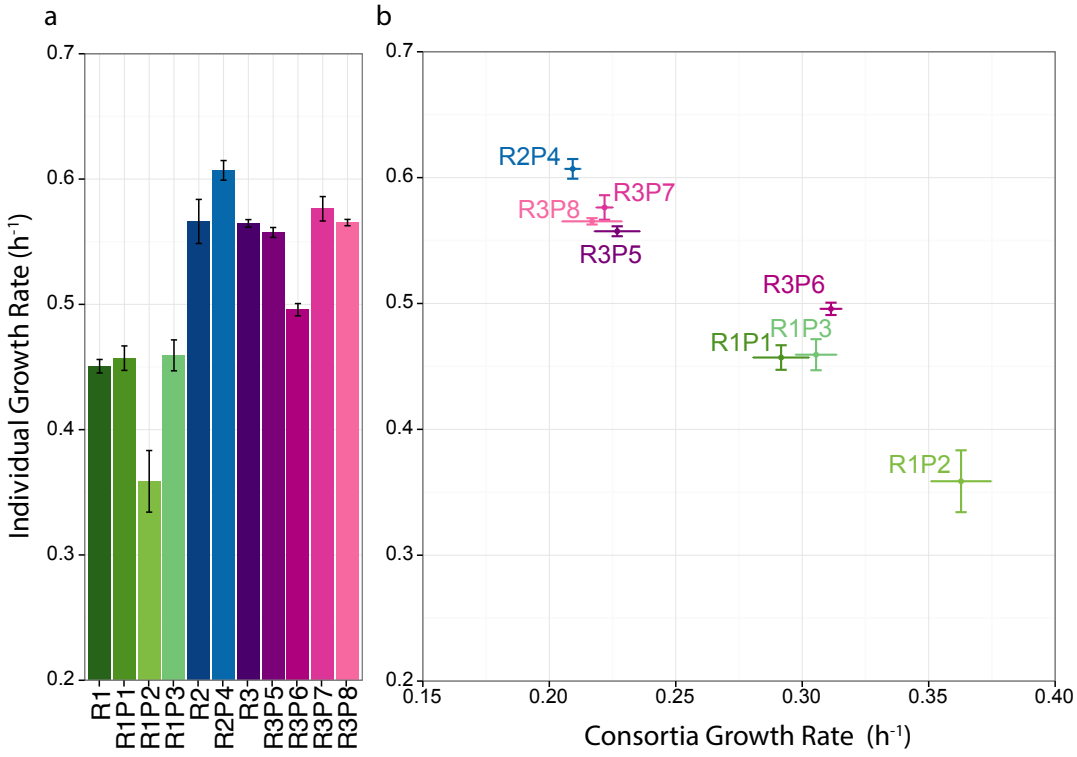

Figure 2.5. Decreases in cooperator individual growth correlates with increases in consortia growth. a) Growth rates of *S. enterica* strains in galactose minimal media show decreased individual fitness in the most cooperative *S. enterica* strains. b) A negative correlation ($R^2 = 0.927$) between individual and consortia growth rates of evolved cooperators demonstrates a trade-off between abiotic and biotic selective pressures in these consortia.

growth, and moderately cooperative R1-derived strains grow similarly to R1, with the most cooperative (R1P2) growing more slowly. The R2 and R3-derived strains exhibited a relatively similar pattern, whereby the most cooperative strain grows most slowly. Examining all the RP cooperators collectively, however, it becomes clear that there is a fairly tight, negative linear correlation ($R^2 = 0.927$) between individual growth on galactose and consortia growth on lactose when methionine production is required (Figure 2.5b). Furthermore, for the most cooperative strain, R1P2, consortia growth rate was indistinguishable from its individual growth rate (Welch t-test, p=0.894). The other cooperators all grew faster individually than when partnered in co-dependence with *E. coli*. Even when excluding R1P2 as an outlier of cooperation, correlation between cooperators' individual and consortia growth rates is maintained ($R^2 = 0.874$).

The most cooperative *S. enterica* strains support highest ratio of *E. coli* partner

Given the wide differences in methionine excretion, individual growth, and community performance, we further examined the consequence of each evolved *metA* allele upon the species ratio in our consortia. *S. enterica* cooperators fluorescently-labeled with yellow fluorescent protein, YFP, were co-cultured with cyan fluorescent protein, or CFP-labeled *E. coli* Δ*metB* in both liquid and solid media. The *E. coli* percentage of each community changed from initial inoculum, reaching equilibria near the end of stationary phase that characterized the community from that point onward (Supplemental Figure 3). *S. enterica* methionine excretion correlates with *E. coli* percentages in liquid culture grown to stationary phase ($R^2 = 0.937$) (Figure 2.6), showing that individual behavior can

predict this ecological trait.

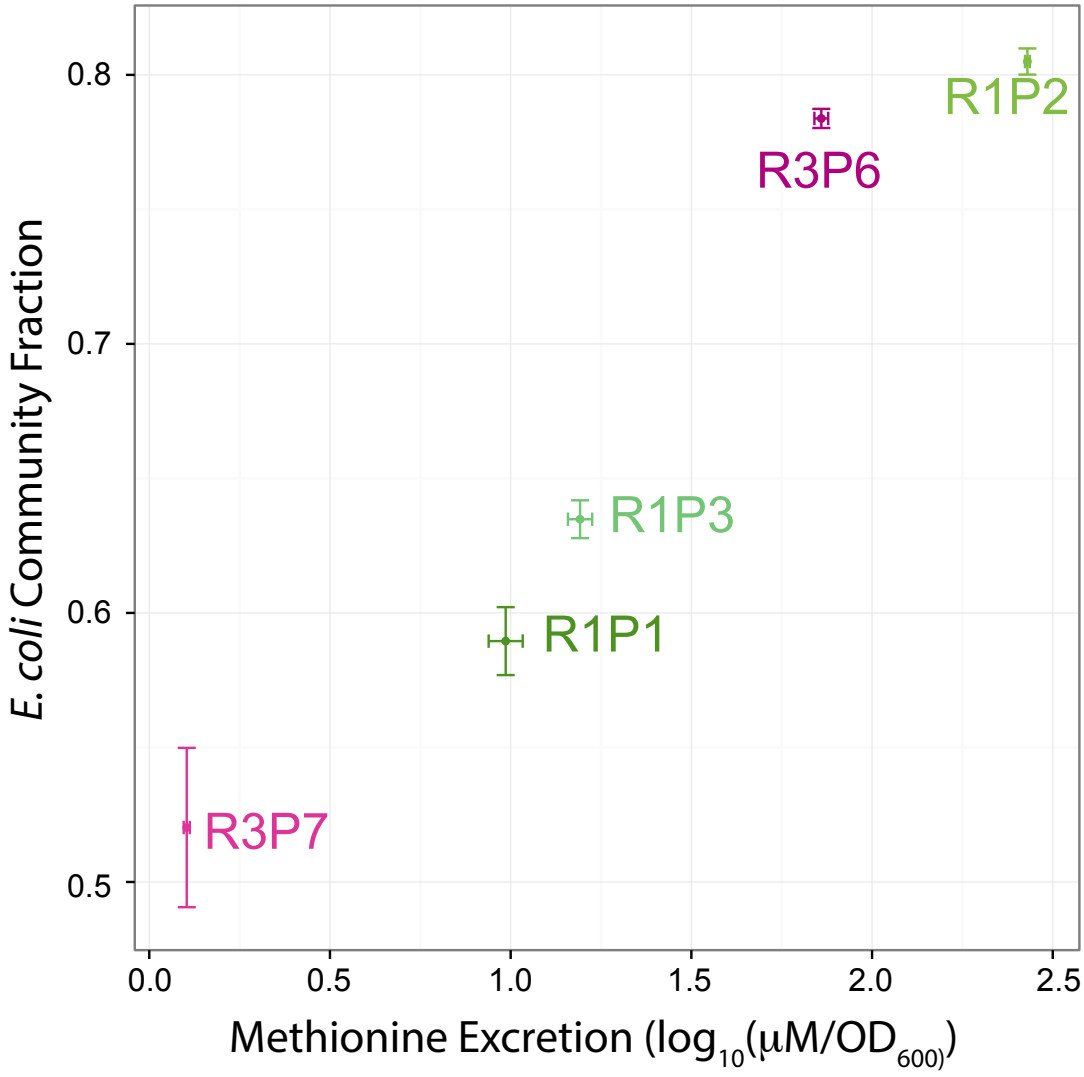

Figure 2.6. **Consortia composition in liquid communities.** YFP-labeled *S.enterica* and CFP-labeled *E. coli* Δ*metB* grown in liquid were sampled at completion of growth, and consortia composition determined via flow-cytometry. *E. coli* composition of each community correlates with methionine excretion of *S. enterica* within that community ($R^2 = 0.937$). Error bars represent standard error of three biological replicates.

Discussion

How much does ecological complexity affect evolutionary repeatability? Within the context of a multi-species, spatially-structured community, the environmental interactions are presumed to be more complex than those faced by individual, planktonic cells in well-mixed media. As such, just as biotic interactions often increase diversification, it might be expected that the distribution of adaptive mutations similarly increase. Our work with a two-species, synthetic consortium suggests quite the contrary: that the initial gateway into a novel interaction may be surprisingly narrow from a genetic perspective. Furthermore, the parallelism observed here was in the absence of competition between newly arising beneficial mutations (i.e., "clonal interference"[22]) that can narrow the winning genotypes to only the best beneficial mutation likely to arise in a given population size.

Why might natural selection have acted so narrowly? After all, chemical selection using methionine analogues leads to a larger diversity of mutation targets within the methionine operon that result in methionine overproduction.[18,23,24] In this case, a relatively dramatic change in phenotype was required for consortia growth, and the ability of consortia to grow was limited by a single factor: methionine production. Given that regulation of methionine production (like many amino acid biosynthetic pathways) is strongly controlled at the first step, HTS encoded by *metA* exerts great control over this phenotype under these conditions. When a single metabolic compound was the currency of exchange, metabolic changes at one point in central metabolism of one organism became responsible for large-scale ecological changes.

An additional factor that may have contributed to the observed genetic parallelism was that the evolved phenotype likely eliminated negative regulation of methionine production. While the work presented here does not directly demonstrate a molecular mechanism, two lines of evidence suggest that methionine overproduction may be due to increased concentration of HTS: previous work demonstrating the N-terminus of HTS that includes several of our P mutations destabilizes the protein[25] and the surprising artifact that arose from our attempts to use a translational fusion to measure HTS concentration.

The N-terminus region of HTS has been shown to play a role in the energy-dependent proteolysis and instability of this enzyme in *E. coli*[25]. Specifically, deletion or replacement of the first 68 amino acids with those of a more stable protein dramatically increases HTS half-life. The *S. enterica* HTS, which shares 95% amino acid identity with the *E. coli* str. K12 enzyme, is likely regulated in the same energy-dependent, proteolytic manner. Six of our eight evolved *S. enterica* HTS amino acid substitutions occur within this N-terminus region, suggesting that the P mutations may increase flux through the methionine biosynthetic pathway by increasing the half-life of HTS. Furthermore, two independent mutations in *metA* that lead to increased heat tolerance in *E. coli*, S61T and I229T[26], both neighbor amino acid substitutions in $metA^{P4}$, $metA^{P5}$, and $metA^{P8}$, suggesting that multiple mechanisms may exist to stabilize this unstable enzyme. Our attempts to directly compare HTS levels between strains via epitope tagging support this idea, as both N- and C-terminus HTS epitope tags simultaneously led to the ancestral strains gaining the cooperative phenotype. Even these slight perturbations resulted in the

methionine overproduction, supporting the idea that a wide range of mutations might increase HTS stability.

This experimental artifact, along with the *E. coli* evidence, suggests that our *S. enterica* adaptations were likely loss-of-function mutations in the domain of the protein that down-regulates HTS half-life, thereby acting as gain-of-function mutations in terms of the pathway to create excess methionine. Given that there is also strict regulation of HTS at the levels of transcription[17], translation[27], and post-translation[25], perhaps these earlier levels of control are simply more difficult to mutate in a single step in order to generate the methionine excretion phenotype needed to have supported consortia growth during our screen. Either way, since many organisms already possess biosynthetic capacities within their cells beyond what they currently export, many organisms may already be primed for the ability to cooperate by simply removing the regulatory restraints.

While the probability and/or the magnitude of beneficial mutations in *metA* led to remarkable genetic parallelism, the resulting alleles conferred a wide spectrum of phenotypes. Genetic parallelism has been a fairly common finding from evolution of microbes in the laboratory, as well as environments such as chronic infections[28], but there are few examples where phenotypic consistency between alleles has been tested[3]. Long-term evolution of *E. coli* has provided a growing list of examples where epistatic interactions differed between various alleles of one gene interacting with other backgrounds (*spoT*[29]; *topA*[30,31]). In our evolved *S. enterica,* neither the mutational location within the enzyme nor ancestral background shows a coherent correlation with resulting methionine excretion. Future studies elucidating the post-translational mechanisms of

HTS control will help bridge our understanding between genetic adaptation and physiological consequence.

Though the link between parallel genetic changes and resulting diverse range of methionine excretion is unclear, our system responded in a predictable and repeatable way at the levels of individual, consortia, and ecological behavior, which all correlated with *S. enterica* methionine excretion. Indeed, we observed a clear tradeoff between individual growth and the positively correlated traits of methionine production and consortia growth rates. This correlation suggests that in the absence of refinement through additional mutations, or of competition between initial mutations to yield a possible Pareto front representing an optimal tradeoff, the metabolite production phenotypes generated by mutations show a consistent trend: the more methionine you make, the slower you grow alone. A second general trend we found was that the more cooperative a strain is, the lower its equilibrium population fraction. This perhaps non-intuitive result is only exacerbated by the above pleiotropy between production and individual growth rates. The most cooperative *S. enterica* strain (R1P2) is illustrative. Its consortia grows exactly as fast as its individual growth rate, suggesting that it is not limited by growth substrate coming from *E. coli*, but that the *E. coli* is solely dependent upon a small, slow population of R1P2 for support. While the paradox of the fastest growing community relying on the slowest growing evolved cooperator might be counterintuitive, it is part of a trend consistent across all repeated evolutions of cooperation in this consortium. Given the methionine output by the *S. enterica* partner, predictions about higher-level community characteristics within this community can be made with some accuracy.

The ability to correlate community composition with future behavior grows increasingly important as we come to rely on genetic sequencing of complex, multi-species microbial systems as a first pass estimator of community character. Accurate inference of ecological behavior becomes more difficult as interactions between shifting selective pressures, genetic changes, and phenotypic expression within an organism are further complicated by species interactions. We have shown here that even highly repeatable genetic adaptations in a dynamic community may translate into an unexpected array of phenotypes; our sequence data alone could not predict the range of observed methionine excretion. Yet consistent evidence of physiological trade-offs and repeatable community dynamics gives some credence to the idea that the trajectory of even a species-rich evolving community might become predictable as our modeling of individual cellular behavior improves.

Materials and Methods

Growth media and strains

The experimental system consisted of an *E. coli* methionine auxotroph (*E. coli* Δ*metB*) and an ethionine-resistant *S. enterica* partner. An *E. coli* strain K12 BW25113 with a *metB* knockout was obtained from the Keio collection, with lactose metabolism restored as described[16]. Ethionine resistant *S. enterica* mutants from LT2 and 14028s backgrounds were selected as described[16]. Cultures were grown in "Hypho" minimal media containing trace metal mix[3] and were supplemented with either 0.1% (liquid media) or 0.05% (solid media) galactose or lactose. Antibiotic concentrations used were: 50 μg/mL ampicillin, 25 μg/mL chloramphenicol (*cat*). All antibiotics and chemicals obtain from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Evolution of methionine excreting *S. enterica* mutants

The two-step selection process for evolving a methionine-excreting *S. enterica* LT2 mutant is described in Harcombe 2010. Initial selection on ethionine, a competitive methionine analog, was again utilized to create new evolutionary ancestors from *S. enterica* serovar Typhimurium 14028s. Co-culturing of ethionine-resistant *S. enterica* and *E. coli* Δ*metB* on lactose Hypho agarose plates to select for methionine excretion proceeded as described[16].

Genomic sequencing

Genomic DNA from *S. enterica* strains LT2, 14028s, and R2P4 was extracted from lysed cells via phenol chloroform extraction[32], and prepared for Illumina sequencing using

TrueSeq kit. Samples were sent to The Microarray and Genomic Analysis Core facility at the University of Utah for sequencing on Illumina HiSeq 2000 sequencer, and aligned and analyzed using breseq[33] (http://www.barricklab.org\breseq) .

Plasmids

Gene replacement utilized the chloramphenicol-containing pKD32 plasmid as a template, and pKD46 as a helper plasmid[34]. 3xFLAG epitope-tagged HTS was constructed using pSUB11[35] as a template. The 5' region of *metA* was cloned into pKNOCK[36] and used as a template for enzymatic inverse PCR[37] construction of Myc and HA epitope-tagged HTS.

Structural analysis of HTS

Homology modeling of HTS (residues 2-297) from *S. enterica* was performed in SWISS-MODEL[38,39] using homoserine transsuccinylase from *Bacillus cereus* (PDB 2h2wA; 49.66% sequence identity) as the template. Alignment of model to 2h2wA, as well as model visualization were performed in MacPyMOL.

Gene Disruption

Gene disruptions were performed using the method of Datsenko and Wanner with modifications described by Ellermeier *et al.*[40]. A selectable chloramphenicol marker (*cat*) flanked by 40 bp of the region surrounding the coding region of *metA* was constructed via PCR using plasmid pKD32 as template[34] and primers listed in Supplemental Table 1. PCR product was cleaned using QiaQuick PCR Purification kit (Qiagen) and electroporated into electrocompetent *S. enterica* cells carrying lambda Red helper

plasmid pKD46. Cells were suspended in LB and recovered for 1 hr shaking at 37 °C before being spread on selective media. Cells were purified once more selectively at 37 °C before Δ*metA*::*cat* insertion was verified via PCR.

P22 Transduction

To create lysates for P22 transduction, *S. enterica* donor strains were grown overnight, and then diluted 1:500 in 5mL LB+cat with 150 μL P22 HT *int* lysate stock and grown with shaking at 37 °C for approximately 6 hours. After vortexing with 1 mL chloroform to kill remaining donor cells and centrifuging 10 minutes at 4550 x *g* to remove debris, lysate was stored at 4 °C for up to 3 years. 200 μL overnight culture of recipient *S. enterica* strains were incubated with 100 μL lysate for 25 minutes at room temperature, rinsed twice with 100 mM sodium citrate LB, plated onto selective media, and grown overnight. After purifying once more selectively at 37 °C, strains were cross-streaked against lytic P22 H5 lysate to test for remaining presence of phage.

Allele Replacement

Native *metA* loci were deleted via P22 transduction of Δ*metA*::*cat* and selection on LB+ chloramphenicol. Cured Δ*metA*::*cat* strains received replacement loci via P22 transduction of donor strains containing the desired new *metA* allele and selection on glucose minimal media.

Epitope Tagging

HTS C-terminus 6xFLAG tag was constructed using pSUB11 as the template, with primers listed in Supplemental Table 1, as described[35]. C-terminus Myc tag and C- and N-terminus HA tags were constructed via EI-PCR[37] using pKNOCK carrying *metA*. All constructs were verified via PCR sequencing before insertion into the *metA* locus of Δ*metA* strains via lambda red recombination. Tagged constructs were amplified and electroporated into R1Δ*metA*::*cat* and R2 Δ*metA*::*cat* electrocompetent cells containing helper plasmid pKD46[41] . Integration into chromosomal *metA* site was selected for on glucose minimal media agarose plates and confirmed via PCR sequencing.

Methionine measurements

Methionine measurements via GC-MS closely followed the method of Zamboni *et al.*[42] To obtain conditioned media samples, overnight cultures of *S. enterica* strains were transferred at a dilution ranging from 50- and 200-fold into 30 mL galactose minimal media, and grown to mid-log phase shaking at 30 °C. Cultures were then pelleted for 10 minutes at 4°C and 4550 x *g,* and then filtered of all cells. After freezing at -80 °C, thawed conditioned media was passed through solid phase extraction Chromaband Easy columns per manufacturer directions (Macherey-Nagel) and eluted in methanol. After removal of methanol in a vacuum centrifuge, and resuspension in 40 μL dimethylformamide, samples were placed into glass vials and derivatized for 1 hour at 85 °C with 40 μL *N*-(tertbutyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1% (wt/wt) tertbutyldimethyl-chlorosilane (Sigma). Derivatized samples were then immediately injected into a Shimadzu QP2010 GCMS (Columbia, MD). The injection source was 230

°C. The oven was held at 160 °C for 1 minute, increased to 310 °C by 20 °C min^{-1}, and held at 310 °C for 30 seconds. Column flow rate was 1.04 mL/min and the split ratio was 1.0. The column was a 30 m Rxi-1ms (Restek, Bellefonte, PA). Results were analyzed in GC-MS Postrun Analysis (Verison 2.70, Shimadzu, Kyoto, Japan). Methionine peak area was compared to an internal standard of added 100 μM isoleucine (Supplemental Figure 4). Experimental methionine concentrations were determined by comparison to known standards, and then divided by the conditioned media culture's final optical density. Methionine concentrations are given as $\mu M/OD_{600}$ and represent a minimum of three biological replicates and three technical replicates.

Growth rate analysis

Strains were acclimated by inoculating single colonies into 640 μL medium in a 48-well microtiter plates and placed in a humidified plate shaking tower (Caliper, Hopkinton, MA) at 30 °C overnight. For growth rate measurements, cultures were then transferred with a 1:1280 dilution into fresh medium and returned to the shaking tower. Optical densities were obtained every 30 minutes (individual growth) or 60 minutes (consortia growth) on a Wallac Victor 2 plate reader (PerkinElmer, Boston, MA) until cultures reached saturation, using an automated measurement system[43]. Growth rates were quantified by fitting the data to a logistic growth model using custom analysis software[44] and averaging a minimum of three biological replicates.

Consortia Composition

S. enterica strains were labeled with a single copy of yellow-fluorescent protein Venus via P22 transduction of the pVenus plasmid at the lambda *attB* site. *E. coli* strain was labeled with a single copy of cyan-fluorescent protein CFP at the ϕ80 *attB* site via P1 transduction. Hypho lactose liquid cultures were inoculated as described above, and 450 μL samples from replicate cultures in the same 48-well plate were collected at inoculation, mid-log, and stationary phase, and frozen at -80 °C in 10% DMSO. 25 mL hypho lactose agarose plates were inoculated with 50 μL of each YFP *S. enterica* strain and 50 μL of the CFP *E. coli* strain and incubated at 30 °C for 72 hours before transfer. To transfer, 1 ml of hypho added to the surface of plates, scraped, vortexed, and then 100 μL was transferred to new plates. Remaining culture from initial inoculation and each subsequent scraping were frozen at -80 °C in 10% DMSO until analysis. Cytometry samples were diluted 1:1000 in PBS and analyzed on BD LSRFortessa II (BD, San Jose, CA). CFP-labeled *E. coli* and YFP-labeled *S. enterica* monoculture and non-fluorescent *S. enterica* were used determine cutoffs for 40,000 events gathered using the 405 nm and 488 nm lasers.

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CHAPTER THREE

Synergistic Epistasis Limits the Evolution of Cooperation between Two Species

Sarah M. Douglas[1,2], Lon M. Chubiz[2], Christopher J. Marx[2,3,4,5*]

[1]Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, United States of America

[2]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts, United States of America

[3]Faculty of Arts and Sciences Center for Systems Biology, Harvard University, Cambridge, Massachusetts, United States of America

[4]Department of Biological Sciences, University of Idaho, Moscow, Idaho, United States of America

[5]Institute for Bioinformatics and Evolutionary Studies, University of Idaho, Moscow, Idaho, United States of America

*Email: cmarx@uidaho.edu

Abstract

Within microbial populations, cross-feeding of costly cellular compounds is a common form of cooperation. When bacteria must overcome multiple levels of metabolic repression to excrete costly compounds, gene interactions like epistasis may limit adaptive strategies. Using an engineered bacterial consortium, we tested how initial selection for transcriptional deregulation affected future evolution of cross-feeding between two species. Without initial selection for resistance to end-product transcriptional inhibition, *S. enterica* struggled to evolve methionine excretion sufficient to support growth of an *Escherichia coli* methionine auxotroph, from whom they required excreted growth substrates. Selection for resistance to a toxic methionine analog, ethionine, enabled more efficient evolution of cooperation by *S. enterica* in a process that required two adaptive mutations. Epistasis between these two mutations, and parallelism at each adaptive step, suggests microbes undergoing *de novo* evolution of cooperation face similar challenges.

Introduction

Resolving the genetic and mechanistic bases of complex biological behaviors remains a central challenge in the post-genomic era. Among these is the emergence of inter-species cooperation, a feature common across levels of biological organization. Of the numerous examples afforded by nature, microbes arguably provide the greatest ability to connect underlying genotypes to cooperative phenotypes. Natural[1–3] and synthetic[4–6] model systems supply information on the end result of natural or artificial selection for cooperation. Yet the evolutionary events preceding the emergence of cooperation remain unclear. Namely, how do the ordering and functional impact of adaptive mutations impact the emergence of the cooperative phenotype?

Epistasis describes when the phenotypic effect of a gene depends upon the genotype at one or more other loci. Such complex interactions are the contours in the adaptive landscape, and thus have the potential to impact the adaption of evolving populations. In the experimental evolution of microbes, whereby evolution can be replayed from the same starting point, it has been commonly found that beneficial mutations provide proportionally less advantage as adaptation proceeds[7–9], although counter-examples exist[10]. Furthermore, which mutations arise first in a lineage have been shown to alter future mutational events[11]. Seemingly subtle differences, such as between multiple beneficial mutations within the same gene, can exert qualitatively different effects upon later mutations[11–13].

Epistasis was observed in our characterization of the *de novo* evolution of cooperation in a synthetic bacterial system, or consortium (Chapter 2). We previously evolved the cross-feeding of costly, mutually-dependent metabolites using a consortium

comprised of two-members: an *Escherichia coli* methionine auxotroph (Δ*metB*) and *Salmonella enterica* serovar Typhimurium[4] (Figure 3.1a). To evolve adaptive methionine excretion by *S. enterica* sufficient to support the *E. coli* partner, we used a two-step selection process. We first selected for *S. enterica* mutants resistant to ethionine, a toxic methionine analog that represses methionine biosynthesis via end-product inhibition, which has previously been used to generate methionine over-production[14,15]. These Eth^R strains, or R strains, acted as starting points for repeated evolution of sufficient methionine excretion by *S. enterica* to support the *E. coli* methionine auxotroph. The second step of the selection process, where one of the R strains was co-cultured with *E.coli* Δ*metB* on lactose minimal media agarose plates, gave rise to an array of cooperative methionine producers, or RP strains. When examining the underlying molecular changes in eight RP cooperators evolved from three R strains, we found that P mutations (denoting methionine production) for all strains were polymorphisms in *metA* (Chapter 2). We showed that mutations in *metA*, which encodes the first enzyme of methionine biosynthesis, were necessary and sufficient for cooperative methionine excretion within the R strains. When placed into the wild-type *S. enterica* background, however, the *metA* alleles were insufficient to generate methionine excretion. The fact neither the R or P mutations alone in the wild-type background can recapitulate methionine excretion indicates synergistic epistasis between these loci.

To test whether the R mutation were necessary for the evolution of cooperation, we attempted to evolve methionine-excreting *S. enterica* without first selecting for ethionine resistance. This evolution proved inefficient but possible in our limited sample size. The adaptive mutation is *metK,* which codes for the enzyme that synthesizes *S*-

adenosylmethionine (SAM), a downstream product of the methionine pathway and co-repressor of methionine pathway genes[16,17]. The phenotype arising from this mutation, however, is less cooperative than those arising from R backgrounds.

We then probed the genetic interactions with R and P mutations in order to identify what molecular mechanism led to increased cooperation in the two-step selection protocol. R1, R2, and R3 all feature mutations at the *metJ* locus, which encodes the methionine pathway repressor, MetJ[18] (Figure 3.1b). Studies in *E. coli* show that MetJ is a transcriptional aporepressor that inhibits methionine synthesis by binding together with SAM to the promoter region. We show that these *metJ* mutations are necessary for cooperative methionine excretion by *S. enterica*, likely through a mechanism of either decreased or eliminated repression of the transcription of the methionine pathway. Together, these *metJ* and *metK* mutational targets lend further credence to the idea that changes in expression patterns often precede changes protein coding sequences in adaptations of new traits[19,20]. Yet the need for two mutations to accrue in the same background to cause robust cooperation illuminates difficulties facing potentially cooperative organisms that must overcome redundant network regulation.

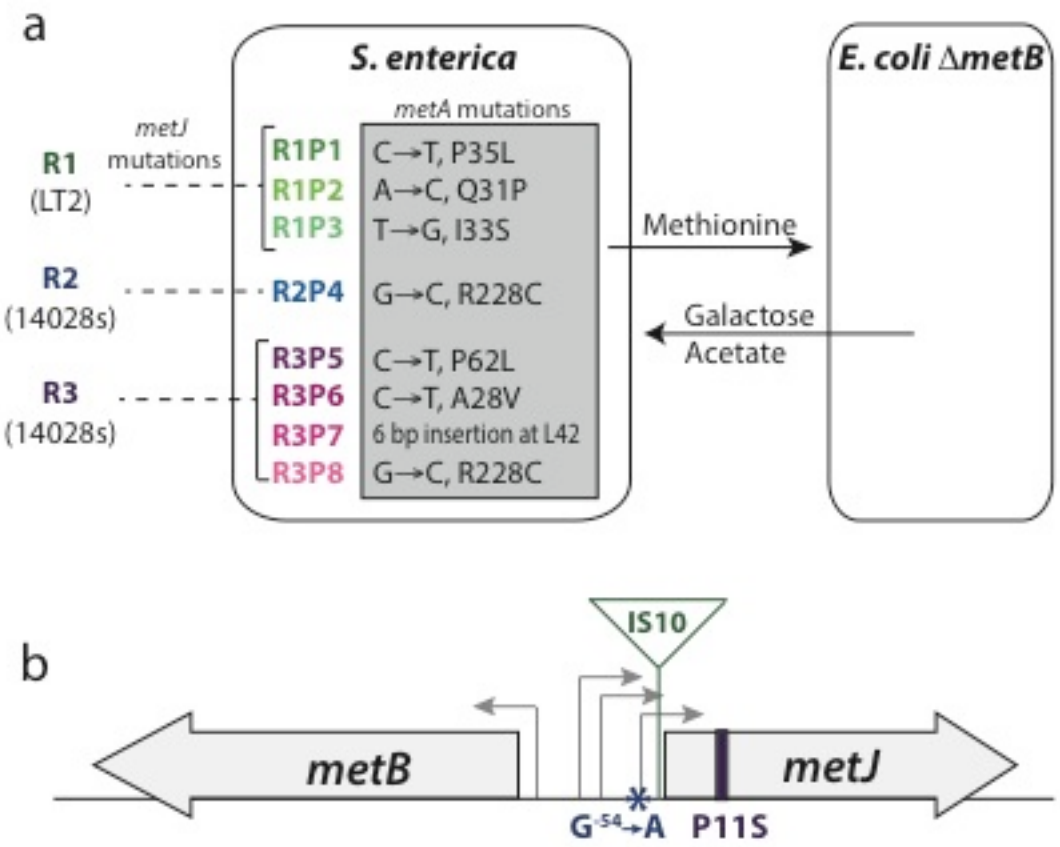

Figure 3.1. **S. enterica producers evolved from ethionine-resistant strains that feature mutations in *metJ*.** a) *S. enterica* ethionine resistant strains (R strains) were evolved from wild-type LT2 and 14028s strains, and then co-cultured with *E. coli* methionine auxotrophs on lactose minimal media. Adaptive methionine excretion by evolved cooperators enabled growth of *E. coli* Δ*metB*, which in turn excretes usable carbon for *S. enterica.* Non-synonymous substitutions that arose in the second selection step within the *metA* gene are listed next to producer strain name. b) A diagram of *metJ* shows the mutations in R1 (IS insertion), R2 (mutation in promoter), and R3 (P11S residue substitution) that arose during the first selection step for resistance to ethionine.

Results

Evolution of cooperation without first selecting for transcriptional derepression is inefficient

Each of our previously evolved *S. enterica* cooperators came from an ethionine-resistant background. To test whether this initial selection for ethionine resistance was necessary, we paired each attempt to evolve cooperation from R strains with attempts to evolve cooperation from wild-type backgrounds. Six attempts proved unsuccessful in the experimental timeframe, so longer time was given. Thus, *S. enterica* LT2 was co-cultured with *E. coli* Δ*metB* on agarose plates for eight weeks, and then diluted and transferred to a fresh plate. For both members of the consortium, lack of cross-fed nutrients did not result in total cell death, merely suspended growth for a subset of surviving cells. Therefore, slow leakage of nutrients from these cells along with potential impurities in media could result in cryptic growth sufficient for adaptation, similar to previous systems[21]. After eight weeks and one transfer, a colony had formed on the agarose plates, similar to those seen previously from R strains that evolved into cooperators. This colony, termed R0P9, contained an *S. enterica* that excreted methionine sufficient to support *E. coli* Δ*metB* growth.

R0P9 excretes levels of methionine per biomas similar to that of other evolved *S. enterica* cooperators like R1P1 (Figure 3.2a). But with a phenotype of slow individual growth, which results in slow consortia growth with co-cultured with *E. coli* Δ*metB*, R0P9's physiological profile differs from the other evolved *S. enterica* cooperators previously evolved from ethionine resistant backgrounds.

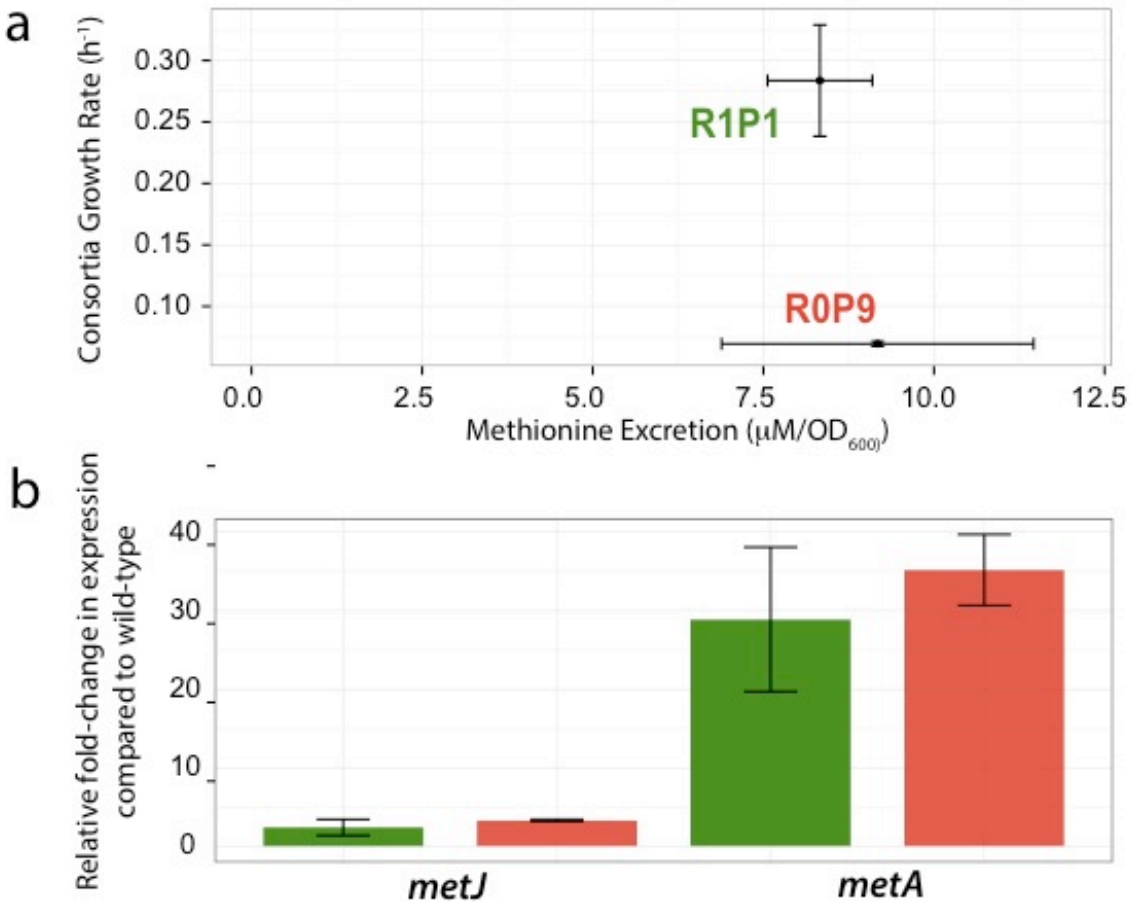

***Figure 3.2*. R0P9 evolved cooperation without first selecting for transcriptional deregulation.** a) Producer R0P9, evolved without first selecting for ethionine resistance, excretes similar amounts of methionine but permits much slower consortia growth compared to R1P1, evolved from ethionine resistance background. b) R1P1 and R0P9 expression similarly increased levels of *metJ* and *metA* compared to LT2 wild-type background. Error bars indicate standard error of three biological replicates.

Whole genome sequencing revealed a non-synonymous mutation in *metK* [Q120L (CAG→CTG)] as the only mutational difference from ancestral *S. enterica* LT2 wild-type, and thus the P mutation for this strain. *metK* encodes the last enzyme in the methionine pathway that catalyzes the formation of *S*-adenosylmethionine (SAM) from methionine. SAM in *E. coli* inhibits the methionine synthetic pathway at both the

transcriptional and translation levels[17,22–24], acting as a co-repressor with MetJ, and likely behaves quite similarly in *S. enterica*[25]. Quantitative RT-PCR data show that *metA* expression, which is normally repressed by MetJ-SAM, is drastically increased by 35.2±9.0 fold in R0P9 relative to its wild-type ancestor, similar to increased expression in the evolved cooperator R1P1 (Figure 3.2b). This increased *metA* expression suggests that the *metK* Q120L substitution may interfere with *metK*'s ability to repress methionine pathway genes via production of SAM.

Cooperators evolved from R strains feature mutations in transcriptional repressor

Where a single-step evolution of cooperation required more time and resulted in a less cooperative *S. enterica* strain, initially selecting for ethionine resistance resulted in an array of evolved cooperators that all outperformed R0P9 in liquid consortia growth (Figure 3.2) (Chapter 2). Whole genome sequencing of *S. enterica* ancestors and cooperators R2P4 and R3P5 (Figure 3.1a) was used to identify genetic changes required for ethionine resistance and subsequent evolution of cooperation. The R2P4 sequence differed from the ancestor by 5 single nucleotide polymorphisms, while R3P5 differed by three (Supplemental Table 2). In addition to *metA*, which is the confirmed causative mutation for the second step of our evolution protocol (Chapter 2), the only other common mutational target was *metJ*, which encodes the methionine operon repressior MetJ (Figure 3.1b). R2P4 contained a $G^{-54}\rightarrow A$ substitution within one of the promoters of *metJ*[26]. R3P5 contained non-synonymous mutation in *metJ*'s coding region, P11S. Subsequent targeted sequencing of *metJ* in R1 revealed another mutation at this locus: an IS*10* insertion 16 nucleotides before the translation start site, in a putative ribosomal

binding site. Thus, all of our evolved cooperators arose from a background that featured a mutation in *metJ*. This locus has been identified as a mutational target in the previous screens for ethionine resistance[18], and sequencing of *metJ* in the ancestral and ethionine-resistant strains of *S. enterica* confirmed that these mutations occurred during selection for ethionine resistance (the R strains) but before the evolution of methionine excretion (the RP strains).

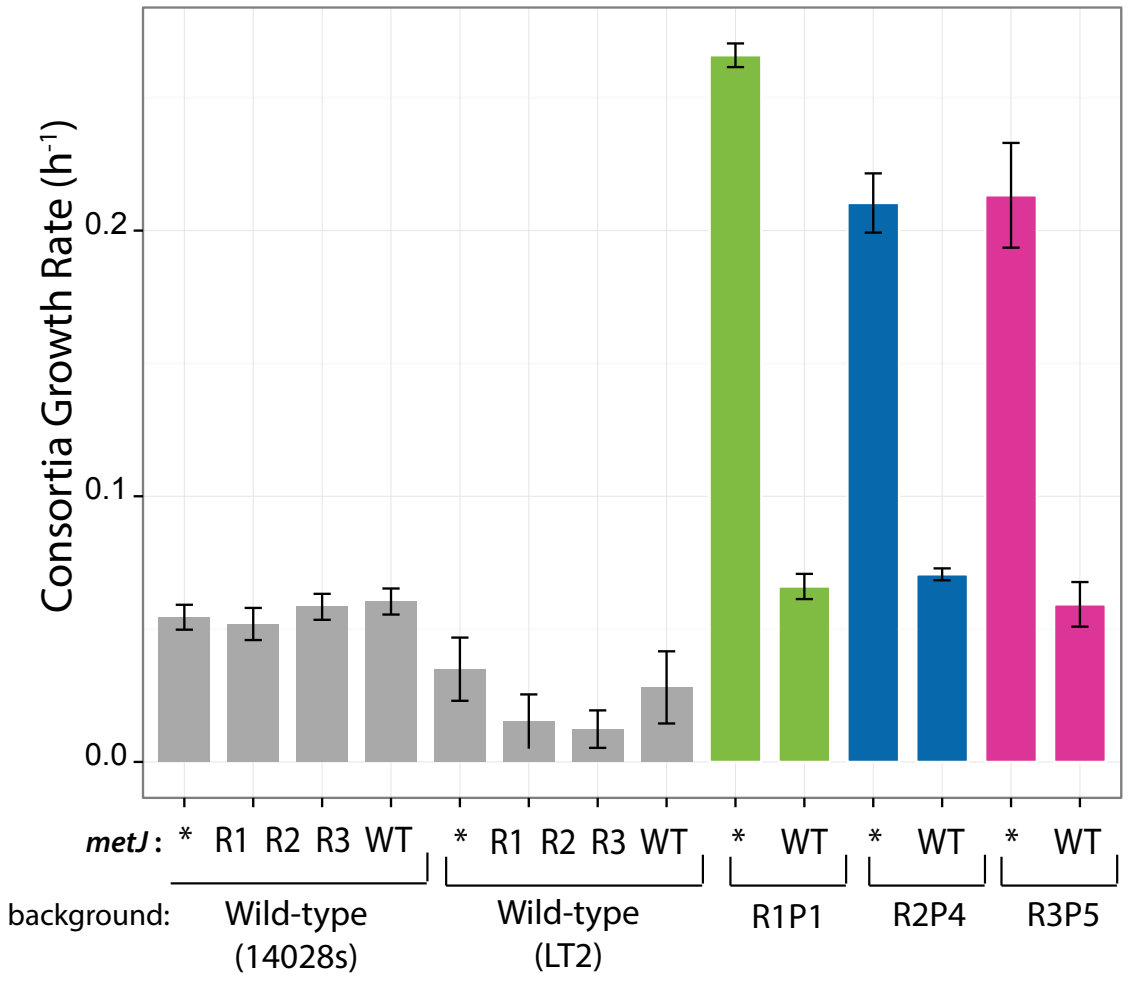

***Figure 3.3*. Ethionine resistant *metJ* is necessary for cooperative phenotype.** Wild-type and Eth^R alleles of *metJ* were substituted into wild-type and evolved *S. enterica*. Asterisks indicate background strain without *metJ* substitution. Insertion of $metJ^{WT}$ into wild-type backgrounds acted as control. Gray bars indicates wild-type background. Error bars indicate standard error of three biological replicates.

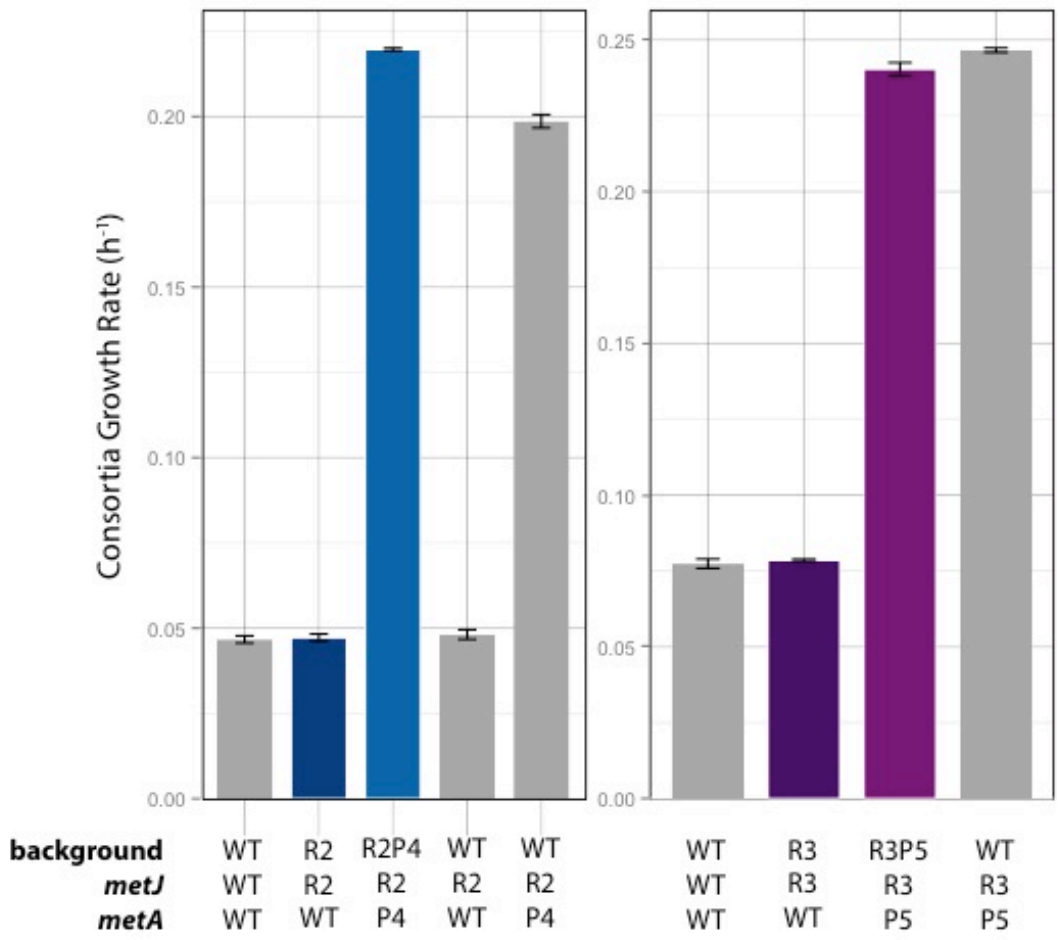

Figure 3.4. **Ethionine resistant *metJ* and evolved *metA* alleles are sufficient to recapitulate cooperative behavior.** Alleles listed in red represent substituted alleles; alleles in black describe the native alleles in that background. Error bars represent standard error of three biological replicates. Gray indicates wild-type background. Differences in wild-type growth rates represent day-to-day variation in growth conditions.

metJ mutations are necessary for cooperation in their evolved backgrounds

Previously engineered strains with the evolved *metA* alleles substituted into the wild-type background failed to excrete methionine, suggesting that R mutations were necessary for cooperation. Indeed, only insertion of evolved *metA* alleles into R strains enabled

methionine excretion (Chapter 2). To directly test whether our *metJ* mutations were a necessary intermediate step in evolution of cooperation, the ancestral $metJ^{WT}$ allele was substituted into cooperator strains and then tested in co-culture with *E. coli ΔmetB*. Evolved *S. enterica* strains with $metJ^{WT}$ failed to cooperate, as did wild-type *S. enterica* with just $metJ^{R1}$, $metJ^{R2}$ and $metJ^{R3}$ (Figure 3.3). Only substitution of both evolved *metJ* and *metA* alleles into ancestral backgrounds recapitulated the evolved cooperator phenotype (Figure 3.4). Thus the *metJ* mutations that arose in the first step of our evolution protocol, along with the *metA* mutations that arose in the second step, are both necessary and, together, sufficient for robust methionine excretion in wild-type *S. enterica*.

metJ mutations decrease individual growth

To quantify the effects of adaptive *metJ* mutations on *S. enterica* growth, individual and consortia growth rates were measured for each WT, R, and evolved strain (Figure 3.5). While the individual growth rate of R2 in galactose minimal media does not significantly differ from that of its ancestral 14028s, both R1 and R3 show a significant decrease in growth compared to their respective wild-type backgrounds. Interestingly, the majority of the cost to individual growth incurred by cooperation does not arise from the *metA* mutation that results in methionine excretion, but rather the *metJ* mutations. R0P9, however, is the slowest growing strain.

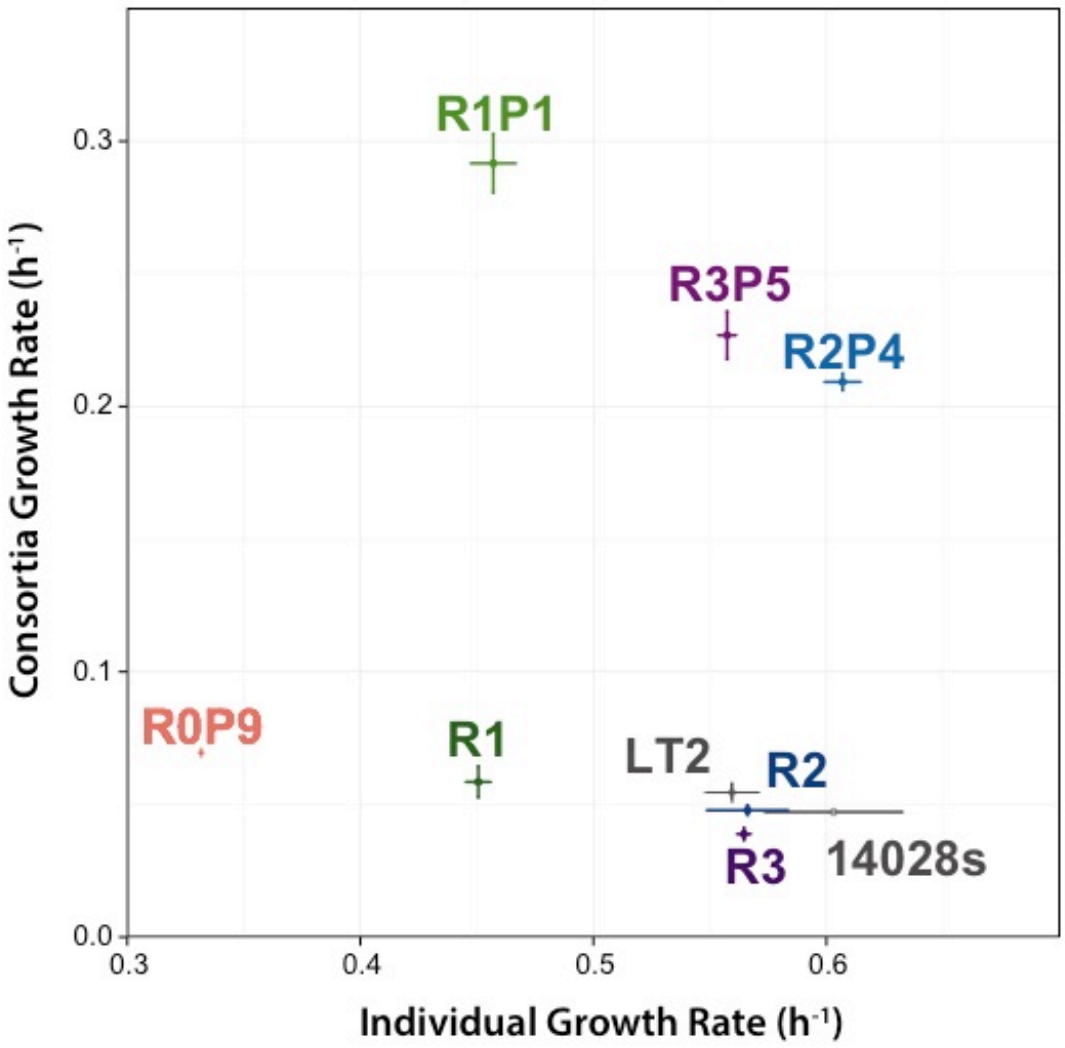

Figure 3.5. **Individual and Consortia growth rates for wild-type, ethionine resistance, and evolved strains.** Individual growth was measured in galactose minimal media, while consortia growth rates represents *S. enterica* strains co-cultured with *E. coli* Δ*metB* in lactose minimal media. LT2 is the wild-type ancestor of R1, which evolved into R1P1. 14028s is the wild-type ancestor of R2 and R3, which each evolved into R2P4 and R3P5, respectively. Error bars represent standard error of three biological replicates.

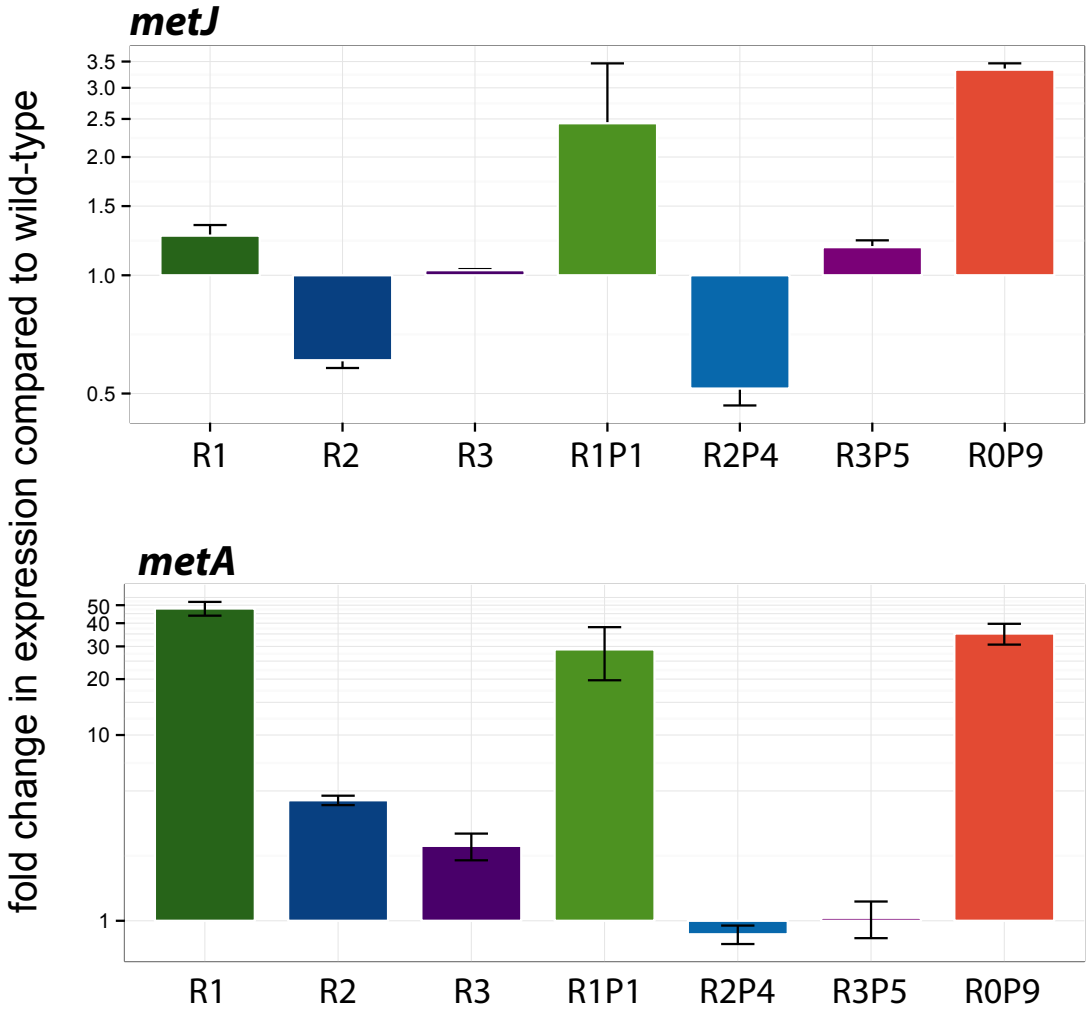

Figure 3.6. **Expression of *metJ* and *metA* in R and RP strains relative to wild-type.** mRNA levels were assayed by quantitative RT-PCR. Error bars represent standard error of three biological replicates.

metJ mutations affect expression patterns in *S. enterica*

Since *metJ* is a transcriptional repressor of methionine pathway genes, including itself and *metA*, we compared expression of these genes in R and RP strains to wild-type (Figure 3.6, Supplemental Table 3). $metJ^{R2}$, which features a promoter mutation, decreased in expression by 39.2 ± 5.4% compared to its wild-type allele. $metA^{R2}$

expression increased 4.54±0.52 fold, likely due to decreased *metJ* expression. $metJ^{R3}$, with a P11S substitution, did not differ significantly in expression from the ancestor, but $metA^{R3}$ expression increased 2.53±0.83 fold. This increase suggests the R3 mutation might functionally inhibit MetJ's repression of methionine pathway genes. The largest change in *metA* expression, however, occurred in R1, which saw a 48±8.17 fold increase. Interestingly, $metJ^{R1}$ did show a slight but significant 26±16% increase in *metJ* expression compared to wild-type. Given that the transposon upstream of $metJ^{R1}$ sits in the middle of a putative Shine-Delgarno sequence (5'-AGGAGGA-3'), $metJ^{R1}$ may be transcribed but not efficiently translated. The transposon insertion may therefore result in a null phenotype, which is supported by R1P1's maintenance of high *metA* expression despite intracellular methionine levels that have likely increased. In contrast, $metA^{R2P4}$ and $metA^{R3P5}$ expression still showed some responsiveness to end-product inhibition by decreasing relative to their R ancestors, returning to wild-type expression levels. This continued responsiveness to intracellular methionine levels suggests that the R2 and R3 *metJ* mutations leave MetJ partly functional. Indeed, the fact that *metA* expression levels in R2P4 and R3P5 do not decrease significantly below wild-type levels despite the high methionine concentration indicates adaptive de-repression of the methionine pathway.

metJ alleles modulate cooperation in non-native background

Although it is clear that the cooperative phenotype required both *metJ* and *metA* mutations, we tested *metJ* alleles in alternative backgrounds to determine whether there was allele-specificity in the combinations that emerged. Cooperation was quantified via measurement of consortia growth rates, and compared to evolved *S. enterica* strains of

the same background (Figure 3.7). *metJ*^{R1} and *metJ*^{R3} have fairly similar impacts on the cooperative phenotype, largely recapitulating various strains' consortia growth when swapped. *metJ*^{R2}, on the other hand, results in decreased consortia growth rates after introduction into a foreign cooperative background. In fact, the evolved R2P4 background, which results in the slowest consortia growth with its native *metJ*^{R2} alleles, increases to become one of the most cooperative strains with a substitution of either *metJ*^{R1} and *metJ*^{R3} (Supplemental Figure 5). This change in consortia growth suggests that both *metA* and *metJ* alleles can generate varying degree of cooperative behavior, but that there is little evidence of specificity between the alleles that emerged together.

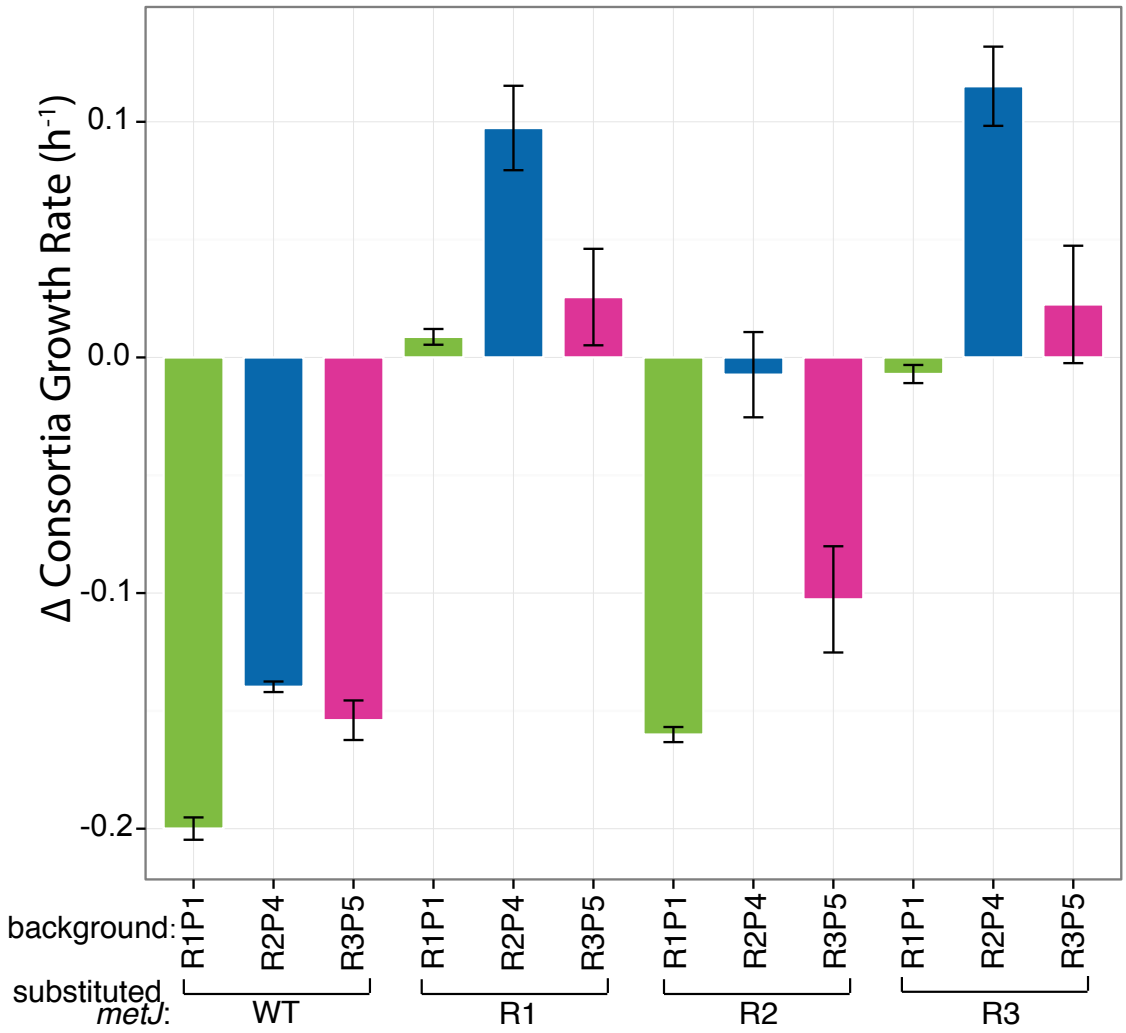

Figure 3.7. **The effect of *metJ* alleles on cooperation in different genetic backgrounds.** Mean growth rate of each cooperator with no *metJ* substitution was subtracted from measured consortia growth rates with substituted *metJ*. Error bars represent standard error of three biological replicates.

metJ deletion mimics R mutations

To test whether loss of *metJ* function allows cooperation, a *metJ* deletion was substituted into wild-type and evolved backgrounds. Like other *metJ* alleles, Δ*metJ* alone does not cause cooperation. However, deletion of this gene, along with $metA^{R1P1}$, is sufficient to recapitulate consortia growth in the *S. enterica* LT2 wild-type background (Figure 3.8),

consistent with the R1 mutation eliminating *metJ* function. Deletion of Δ*metJ* in the 14028s wild-type background also results in methionine excretion when paired with an evolved *metA* allele. Given that Δ*metJ* mimics the phenotype of R2 and R3 mutations in both wild-type and evolved strains, the functional impact of these R mutations likely produces a significant down-regulation or loss of function in *metJ*. Thus, a variety of mutations targeting *metJ* can generate a background conducive to evolution of cooperation.

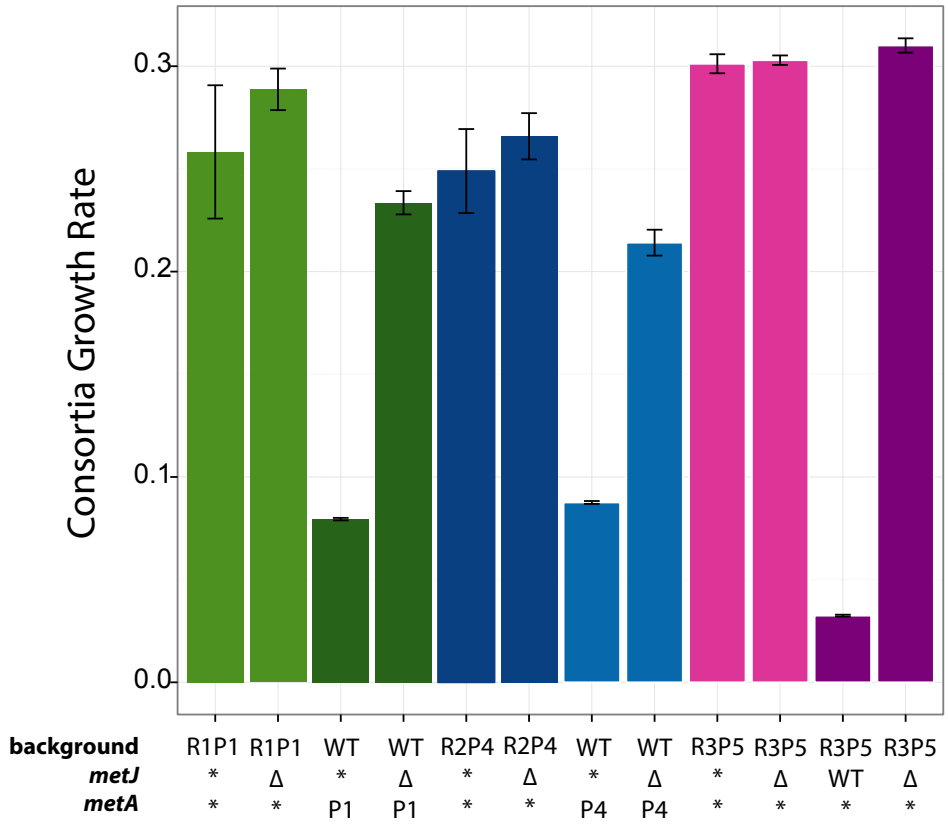

Figure 3.8. Null *metJ* mutant and evolved *metA* alleles allows cooperation. Asterisks indicate native allele (no substitution). Δ*metJ* induced cooperation in wild-type backgrounds containing $metA^{R1P1}$ and $metA^{R2P4}$. Additionally, Δ*metJ* rescued cooperation in evolved R3P5 background with $metJ^{WT}$.

Discussion

Cooperation through sharing of small, costly cellular commodities can be found in natural microbial populations[1–3]. When shared commodities are costly to produce, explaining the origin of such behavior becomes challenging from both an evolutionary and a mechanistic viewpoint. Broadly, how do these costly adaptations spread in a population if they increase the fitness of unrelated individuals? More specifically, how do cooperative individuals untangle redundant layers of repression that normally regulate the production of commodities costly to the cell?

Using two selection strategies, we probed possible evolutionary trajectories leading to cooperation in the form of methionine overproduction by *S. enterica*. Initial evolution of cooperation without selecting for ethionine resistance, mimicking the conditions more likely to be experienced in natural systems during *de novo* evolution of cooperation, reached a successful cooperative phenotype in a single mutation that resulted in excretion of high levels of methionine per biomass. Yet selective conditions did not require fast growth, and poor individual growth rate resulted in a slow consortium growth rate. Given the magnitude of the *metA* expression increase, this cooperator's causative mutation $metK^{P9}$ could decrease enzymatic activity and slow the production of MetJ's co-repressor. Interestingly, though R0P9 is a product of natural selection in our consortium, *metK* has previously been identified in chemical mutagenesis of *S. enterica* via methionine analogs[23]. Therefore, similar mutation targets can arise from natural and chemical selection for derepression of transcription of the methionine pathway. The cooperator resulting from this selection pressure to solve methionine cooperation in a single mutation, however, still falls short of those allowed to evolve in two steps.

Evolving cooperation after first selecting for ethionine resistance greatly improved both the efficiency and strength of the resulting cooperation. In pathways like methionine biosynthesis that are regulated at the levels of transcription, translation, and post-translation, a single mutation is unlikely to overcome all levels of redundant repression. Thus, initial selection for ethionine resistance before co-culturing with the *E. coli* partner allowed two mutations to accrue in the same background. Mutations in the transcriptional repressor *metJ* resulted in increased expression of methionine pathway genes, like *metA*. From this background evolved *metA* mutants that feature non-synonymous substitutions or insertions in the coding region of *metA*'s protein, HTS, which likely increased protein stability (Chapter 2). Alone, each mutation is unable to overcome repression at the other level of regulation. Together, they are able to decouple the rate of methionine synthesis from cellular methionine concentration. This temporal ordering where transcriptional regulation is targeted first is a common finding in metabolic engineering, and the evolution of enzymes. Though examples do exist where changes in coding sequence proceeded changes in expression in evolving populations[27], regulatory mutants generally precede structural ones in enzyme evolution, especially if the enzyme is very efficient[28].

This degree of epistasis highlights the challenge faced by potentially cooperative bacteria evolving overproduction of tightly regulated cellular commodities. These R mutations are not beneficial on their own in the environment. Thus they will exist at low levels due to mutation-selection balance, but would only be enriched in an environment such as one containing ethionine where derepression is beneficial. Other evolutionary paths to methionine overproduction may be available to *S. enterica*, but parallelism at

both steps of adaptation here show all eight evolved *S. enterica* followed the same adaptive solutions. These two stages of parallelism are particularly noteworthy because the selection of ethionine-resistant colonies or cooperative consortia both occurred by directly visualizing a beneficial event in that environment. This therefore eliminates competition between lineages, known as clonal interference[29], which commonly sorts for the beneficial mutations of the greatest effect. This sorting can lead to remarkable parallelism between and within populations[30].

Overall, in our evolution of cooperation, epistatic interactions between adaptations necessitated a specific ordering of selective pressures in order to efficiently arrive at our cooperative phenotype. In the evolution of other types of cooperative cross-feeding, a similar ordering of adaptations may likely occur if more than one mutation is necessary. If the currency of exchange are costly compounds like amino acids and vitamins, which are also tightly repressed via end-product inhibition[31], the breaking of regulation may be generally available as a strategy to become a cooperator, but may be constrained genetically to require multiple interacting mutations.

Methods and Materials

Growth media, strains, and plasmids

The experimental system consisted of an *E. coli* methionine auxotroph (*E. coli* Δ*metB*) and an ethionine-resistant *S. enterica* partner. An *E. coli* strain K12 BW25113 with a *metB* knockout was obtained from the Keio collection, with lactose metabolism restored as described[4]. Ethionine-resistant *S. enterica* mutants from LT2 and 14028s backgrounds were selected as described[4]. Cultures were grown in "Hypho" minimal media containing trace metal mix[32] and were supplemented with either 0.1% (liquid media) or 0.05% (solid media) galactose or lactose. Gene replacement utilized the chloramphenicol-containing pKD32 plasmid as a template, and pKD46 as a helper plasmid[33]. Antibiotic concentrations used were: 50 μg/mL ampicillin, 25 μg/mL chloramphenicol (*cat*). All antibiotics and chemicals obtain from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Evolution of methionine excreting *S. enterica* mutants

The two-step selection process for evolving a methionine-excreting *S. enterica* LT2 mutant as described[4]. Initial selection on ethionine, a competitive methionine analog, was again utilized to create new evolutionary starting points in *S. enterica* serovar Typhimurium 14028s. Co-culturing of ethionine-resistant *S. enterica* and *E. coli* Δ*metB* on lactose minimal media agarose plates to select for methionine excretion proceeded as described[16]. Evolution of cooperator R0P9 arose from co-culture of 500 μL of *S. enterica* LT2 grown to saturation in hypho glucose and 500 μL of *E. coli* Δ*metB* grown in hypho lactose with 100 μM methionine, pelleted, resuspended in minimal media, spread onto a

lactose hypho agarose plate, and transferred after 8 weeks of growth at 30 ˚C to isolate new cooperative colony at a 1:10 dilution.

Genomic sequencing

Genomic DNA from *S. enterica* wild-type strains LT2 and 14028s, and our evolved *S. enterica* strains R3, R2P4, R3P5, and R0P9 was extracted from lysed cells via phenol chloroform extraction[34], and prepared for Illumina sequencing using TrueSeq kit. Samples were sent to The Microarray and Genomic Analysis Core facility at the University of Utah for sequencing on Illumina HiSeq 2000 sequencer, and aligned and analyzed using breseq[35] (http://www.barricklab.org\breseq) .

Gene Disruption

Deletion of *metJ-metB* were performed using the method of Datsenko and Wanner with modifications described by Ellermeier *et al.*[36]. A selectable chloramphenicol marker (*cat*) flanked by 40 bp of the region surrounding the coding region of *metJ-metB* was constructed via PCR using plasmid pKD32 as template[33] and primers listed in Supplemental Table 4. PCR product was cleaned using QiaQuick PCR Purification kit (Qiagen, Valenica, CA) and electroporated into electrocompetent *S. enterica* cells carrying lambda Red helper plasmid pKD46. Cells were suspended in LB and recovered for 1 hr shaking at 37 °C before being spread on selective media. Cells were purified once more selectively at 37 °C before Δ*metJB*::*cat* insertion was verified via PCR.

P22 Transduction

To create lysates for P22 transduction, *S. enterica* donor strains were grown overnight, and then diluted 1:500 in 5 mL LB+cat with 150 μL P22 HT *int* lysate stock and grown with shaking at 37 °C for approximately 6 hours. After vortexing with 1 mL chloroform to kill remaining donor cells and centrifuging 10 minutes at 4550 x *g* to remove debris, lysate was stored at 4 °C for up to 3 years. 200 μL overnight culture of recipient *S. enterica* strains were incubated with 100 μL lysate for 25 minutes at room temperature, rinsed twice with 100 mM sodium citrate LB, plated onto selective media, and grown overnight. After purifying once more selectively at 37 °C, strains were cross-streaked against lytic P22 H5 lysate to test for remaining presence of phage.

Allele Replacement

Native *metJ/metB* loci were deleted via P22 transduction of Δ*metJ/metB*::*cat* and selection on LB+chloramphenicol. Cured Δ *metJ/metB* strains received replacement loci via P22 transduction of donor strains containing the desired new *metJ/metB* locus and selection on glucose minimal media. *metB*, located within the 1.4 kb region downstream of *metJ*, was included in this deletion to allow growth in minimal media to be used as a counter-selection against allele replace. *metJ* is not necessary for growth in these conditions, and Δ*metJ* strains are indistinguishable from successful transformants in this selection.

Growth rate analysis

Strains were acclimated by inoculating single colonies into 640 µL medium in a 48-well microtiter plates and placed in a humidified plate shaking tower (Caliper, Hopkinton, MA) at 30 °C overnight. For growth rate measurements, cultures were then transferred with a 1:1280 dilution into fresh medium and returned to the shaking tower. Optical densities were obtained every 30 minutes (individual growth) or 60 minutes (consortia growth) on a Wallac Victor 2 plate reader (PerkinElmer, Boston, MA) until cultures reached saturation, using an automated measurement system[37]. Growth rates were quantified by fitting the data to a logistic growth model using custom analysis software[38] and averaging a minimum of three biological replicates.

Quantitative RT-PCR

10 mL cultures were grown to early log phase (OD_{600} = 0.10-0.12) in galactose minimal media, and pellets were snap frozen in liquid nitrogen before storing at -80 °C. Cells were lysed with RNAse Free Lysis Matrix beads (MP Biomedicals, Santa Ana, CA) and RNA was extracted using an RNAeasy Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, using gene specific primers listed in Supplemental Table 4. qPCR was performed using fast EvaGreen PCR Master Mix (Biotium, Hayward, CA) and quantified on a CFX384 Touch Real-Time PCR Detection System (BioRad, Hercules, CA). *metA* and *metJ* gene expression values for each strain represent three biological replicates, each of which is composed of three technical replicates. Relative quantification proceeded as described[39],

using *gryB* as reference gene. Standard curves for each primer set, as well as no RT and no template controls, were included on the same plate as experimental samples.

Methionine measurements

Methionine measurements via GC-MS closely followed the method of Zamboni *et al.*[40] To obtain conditioned media samples, overnight cultures of *S. enterica* strains were transferred at a dilution ranging from 50- and 200-fold into 30 mL galactose minimal media, and grown to mid-log phase shaking at 30 °C. Cultures were then pelleted for 10 minutes at 4°C and 4550 x *g,* and then filtered of all cells. After freezing at -80 °C, thawed conditioned media was passed through solid phase extraction Chromaband Easy columns per manufacturer directions (Macherey-Nagel, Bethlehem, PA), washed with ddH_20, and eluted in methanol. After removal of methanol in a vacuum centrifuge, and resuspension in 40 μL dimethylformamide, samples were placed into glass vials and derivatized for 1 hour at 85 °C with 40 μL *N*-(tertbutyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1% (wt/wt) tertbutyldimethyl-chlorosilane (Sigma). Derivatized samples were then immediately injected into a Shimadzu QP2010 GCMS (Columbia, MD). The injection source was 230 °C. The oven was held at 160 °C for 1 minute, increased to 310 °C by 20 °C min^{-1}, and held at 310 °C for 30 seconds. Column flow rate was 1.04 mL/min and the split ratio was 1.0. The column was a 30 m Rxi-1ms (Restek, Bellefonte, PA). Results were analyzed in GC-MS Postrun Analysis (Verison 2.70, Shimadzu, Kyoto, Japan). Methionine peak area was compared to an internal standard of added 100 μM isoleucine. Experimental methionine concentrations were determined by comparison to known standards, and then divided by the conditioned

media culture's final optical density. Methionine concentrations are given as mM/OD_{600} and represent a minimum of three biological replicates and three technical replicates.

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CHAPTER FOUR

Conclusion

Using a tractable bacterial system that allows the *de novo* evolution of cooperation, I was able to explore the molecular mechanism underlying community change. Repeatedly replaying the evolution of cooperation between my *S. enterica* and *E. coli* partners yielded a range of cooperative strategies. I quantified differences in behavior at the individual and community levels within this spectrum, and determined that methionine excretion was the variable that could clearly predict higher-level behavior, like community composition. Such predictive power likely arose due to trade-offs between individual and community-level factors created by the cost of methionine excretion. Examination of the molecular mechanism underlying this cooperation revealed mutations in *metA* and *metJ* characterize each cooperator evolved in using a two-step evolution protocol.

The genetic parallelism observed in this system provides an exciting opportunity to link the changes in community behavior to a single gene. Efforts to model multi-species microbial communities are currently hampered by the inability to predict interactions between component parts[1], though some successes have been achieved in predicting pair-wise interactions within the same species[2]. Just as the behavior of individual cells can be modeled without knowledge of each and every gene, so too could behavior of microbial communities be inferred without full genetic sequences of all individuals if key mutation targets could be identified and linked to community change.

The structure of my evolutionary protocol raised questions about historical contingency, which is believed to play a large role in shaping evolution. *S. enterica* strains resistant to ethionine, a toxic methionine analog, more efficiently and successfully evolved cooperation than those without previous exposure to ethionine. Thus, the

potential for entering into cooperation may be contingent on what previous selective pressures an organism has experienced – not an uncommon occurrence in evolution. As a process that samples solutions without replacement, evolution renders any surviving lineage a living record of past selective forces. The role of historical contingency in this consortium, however, raises concerns for likelihood of similar adaptive trajectories in natural populations. When costly cellular commodities are the currency of exchange in a cooperative relationship, bacteria must overcome layers of regulation likely repressing the production of such commodities. Epistasis arising from this redundancy of regulation suggests that more than one mutation may need to accrue in a single background before this type of altruism can occur.

Future directions

In future work on my specific evolved cooperators, confirming the causal relationship between *metA* mutations and HTS protein stability would provide the final mechanistic link between genotype and ecology in these consortia. Due to its low native level of expression, and the success of previous studies using translational fusions[3], an anti-HTS antibody was deemed inadvisable early in this project. After four different epitope tagging constructs induced cooperation as an artifact in ancestral backgrounds, and a failed attempt directly probe HTS levels in ancestral and evolved strains by examining *S. enterica*'s proteome via liquid chromatography-mass spectrometry[4], a Western blot assay using an anti-HTS probe may be, in retrospect, the best method.

Another logical future experiment would be to examine the consequences of each *S. enterica* strain's cooperative strategy in long-term evolution experiments on liquid and

solid media. Previous long-term evolution of cooperative populations has seen the rise of cheaters, compensatory mutations to ameliorate costly cooperation, and the rise of stable polymorphisms[5–10]. By examining a spectrum of cooperative strategies over time, rather than just one strategy, my consortia could help elucidate how the extent of commitment to cooperation influences these scenarios. In addition, this long-term evolution would allow observation of the *E. coli* partner's adaptive response to each *S. enterica* cooperative strategy. Previous long-term evolution of R1P1 consortia saw the rise of an interesting adaptive response in the *E. coli*: a mutation in *galK* that increased the *E. coli*'s output of galactose, which in turn increases *S. enterica* growth (Harcombe and Marx, unpublished data). Would such an adaptation be more likely to arise with a more cooperative partner, like R1P2, or to increase numbers of a poor cooperator, like R2P4? This consortium is a two-player game, and a long-term evolution experiment would allow *E. coli* Δ*metB* a chance to respond to *S. enterica*'s adaptive strategies.

Third, though the work presented here characterized the range of cooperative strategies represented by the evolved *S. enterica* strains, it does not provide the ability to rank them. Which balance between the opposing biotic and abiotic selective pressures represents the most fit strategy in this consortium? Competing pairs of *S. enterica* cooperators, or all eight together, in consortia with *E. coli* Δ*metB* and measuring the frequency of each over time would determine the optimal cooperative strategy. Furthermore, comparing the results of these competitions between cooperators in liquid versus solid media could help illuminate how reciprocity impacts social behaviors in natural systems. Differentiating eight different fluorescent markers for each *S. enterica* strain is currently beyond the capability of most flow cytometry technology. Instead,

utilizing deep-sequencing to measure allele frequency of barcoded strains through time[11] has the potential to provide highly accurate measurements of relative strain proportions in evolving, multi-cooperator consortia.

Lastly, in order to expand the predictive power of my conclusions, it would be interesting to attempt evolution of this cooperation in a species other than *S. enterica*. The same strategy of evolving resistance to a methionine analog, and then co-culturing the *E. coli* Δ*metB* partner, should be feasible in other bacteria[12]. If the parallelism observed in *S. enterica* holds for evolved cooperators from other species, then expectations of similar patterns in cooperation populations could help predict community behavior. Initial attempts to evolve new methionine cooperation using an engineered *E. coli-Shewanella oneidensis* consortium were unsuccessful, but by no means exhaustive. Exploration of other bacterial partners, and cooperation using other amino acids or cellular products like vitamins and nucleotides, would elucidate the limits and potential of the predictive patterns observed in *S. enterica-E. coli* consortia.

Industrial applications

Evolving such new obligate mutualisms in bacterial consortia also has interesting industrial applications. The extent of methionine excreted by the most cooperative *S. enterica* strains suggests that selection in synthetic communities may serve as a viable path to obtaining industrially-relevant strains. Growth in a co-dependent, structured community accomplishes what is usually a challenging task: how to associate a genotype's fitness with production of a desired metabolite. Indeed, methionine is a valuable commodity to microbes and men alike, with numerous patents claiming

intellectual property over mutations in *metA*[13,14]. Published values of methionine excretion by engineered *E. coli* strains are similar to those of the most cooperative of our evolved *S. enterica* strain[15]. The Δ*thrBC*, Δ*metJ* engineered strain containing a *metK* substitution and a combination of three *metA* mutations excreted 5.9 mg of methionine per gram of glucose in medium. *S. enterica* producer R1P2 excreted 1.2 mg of methionine per gram of galactose in medium, and was collected mid-log phase before growth was complete. In our *S. enterica* producers, experimental evolution repeatedly arrived at an efficient answer to the problem of costly overproduction. This suggests that, when properly applied, selective pressure can work as an efficient engineer of desired metabolic traits.

Fin

I hope the work presented here contributes in small part to the vibrant and expanding frontier of microbial evolution. Even with a data revolution following the genomic, tractable biological systems will still be needed more than ever to correct and correlate models to real-world behavior. Improving such models will, in turn, allow for increasingly accurate predictions about the behavior of evolving communities. Such a virtuous cycle represents a kind of cross-feeding that, similar my consortia, has the potential to grow more interdependent and productive over time, achieving greater yields than either half alone.

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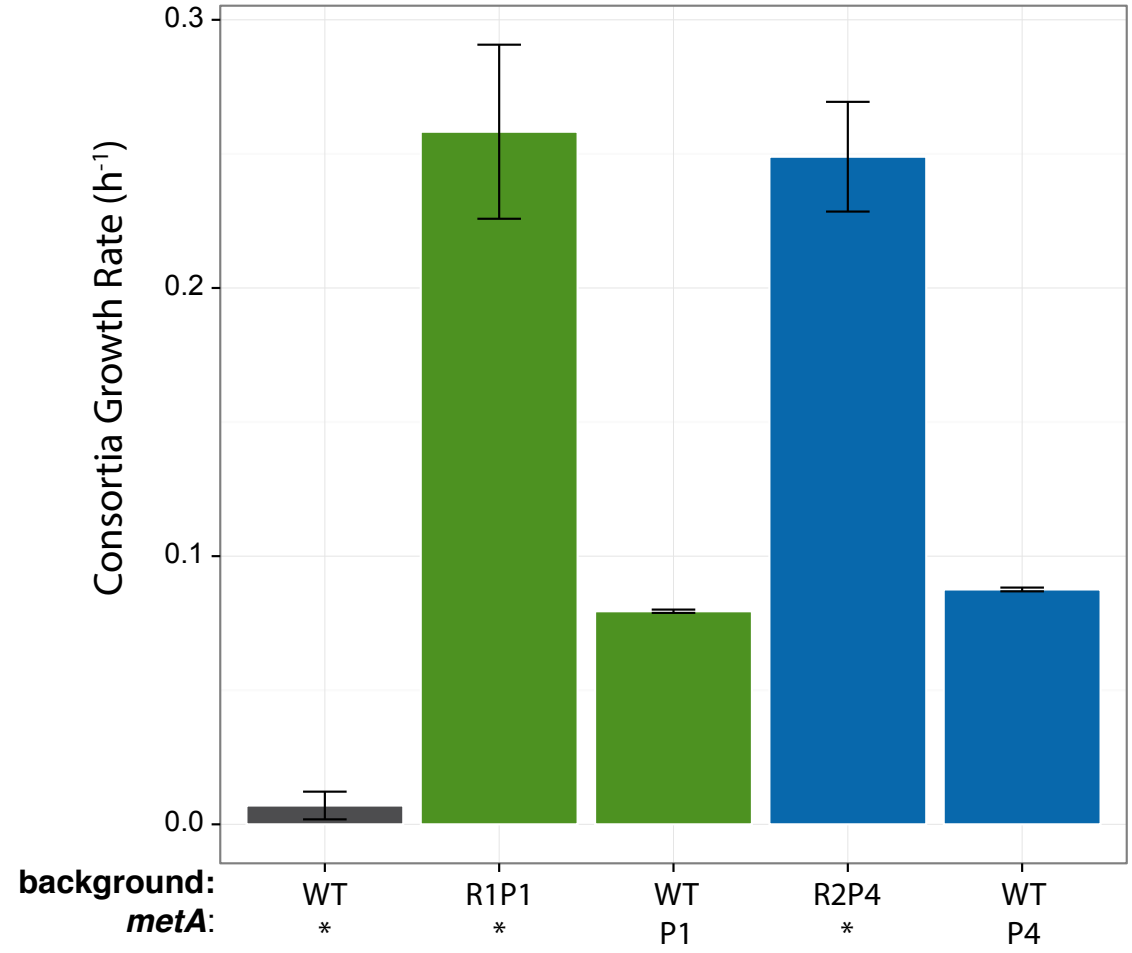
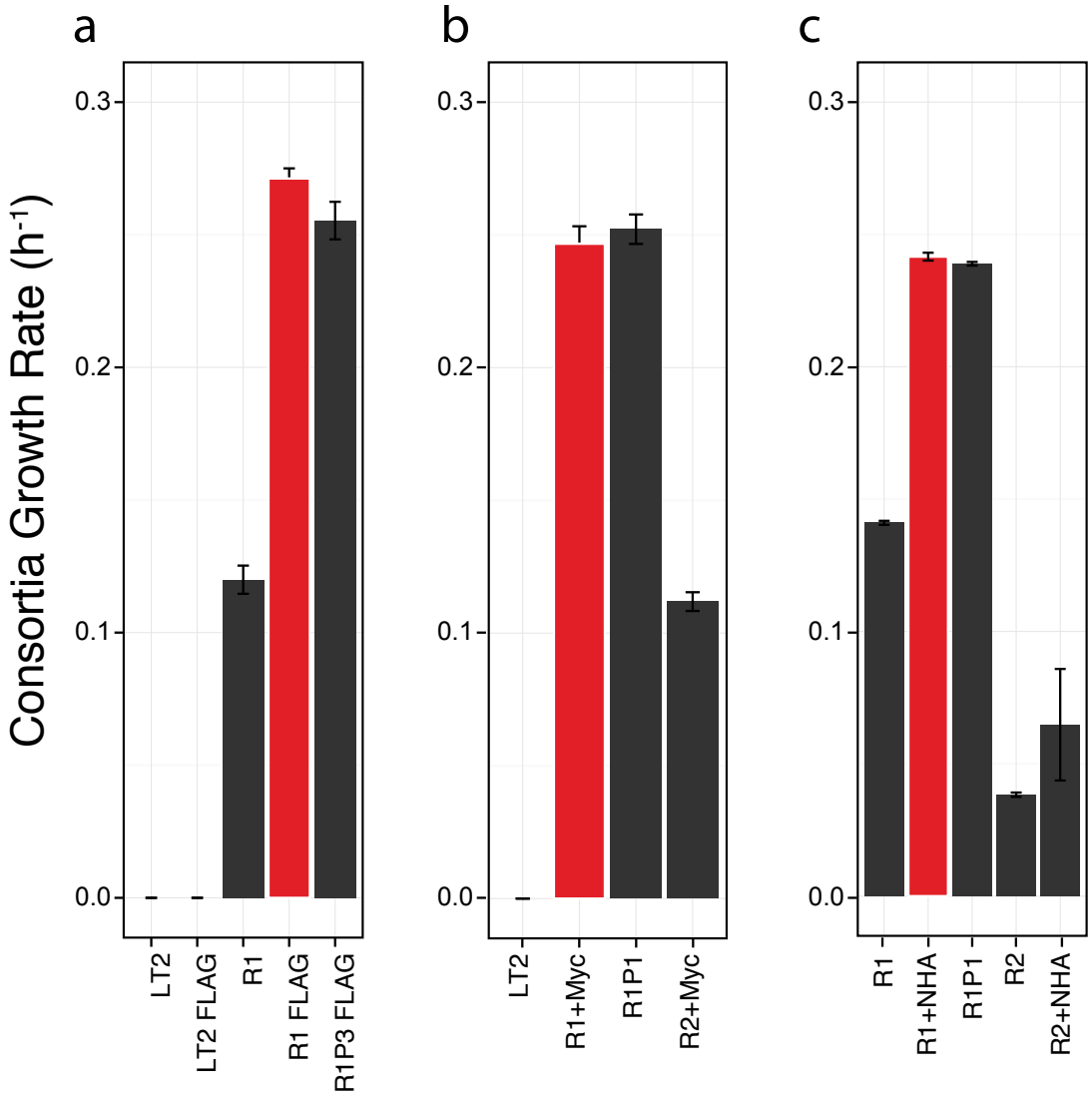
APPENDIX

Supplemental Figures and Tables

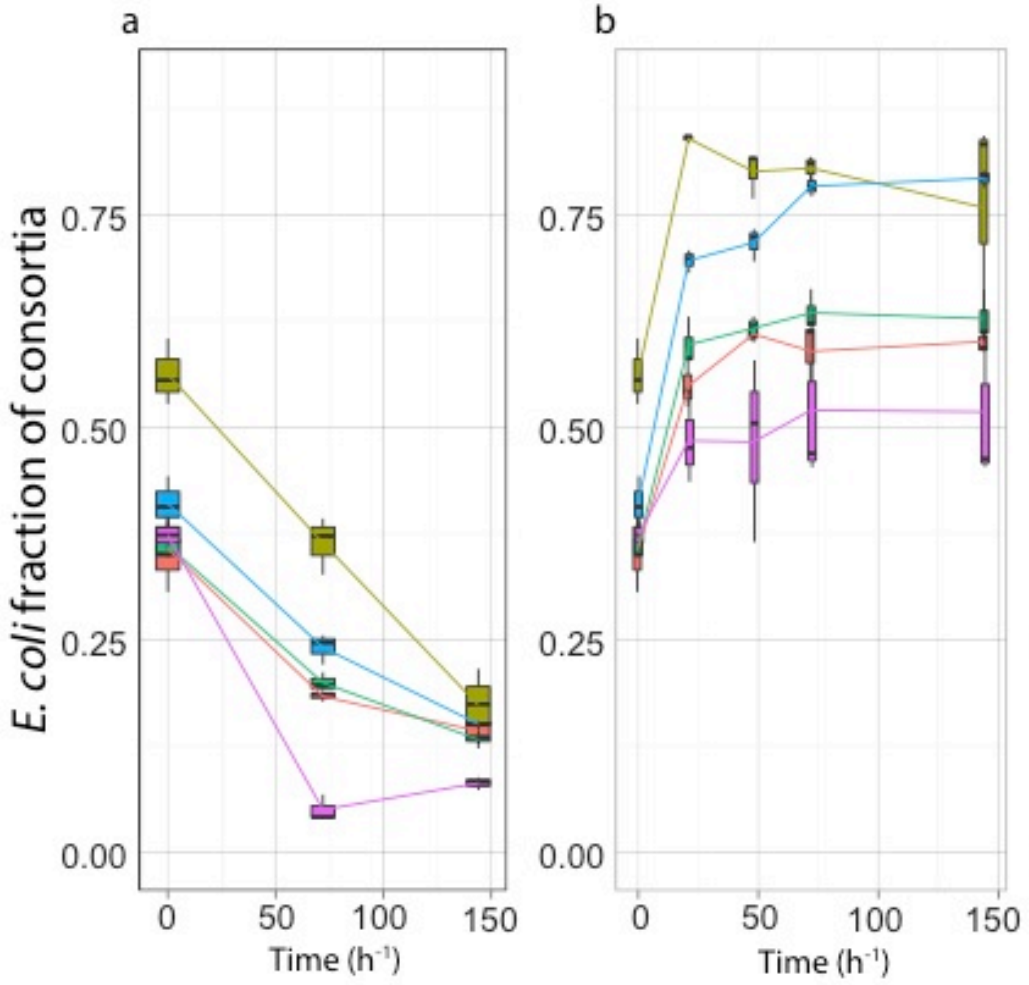
Supplemental Figures



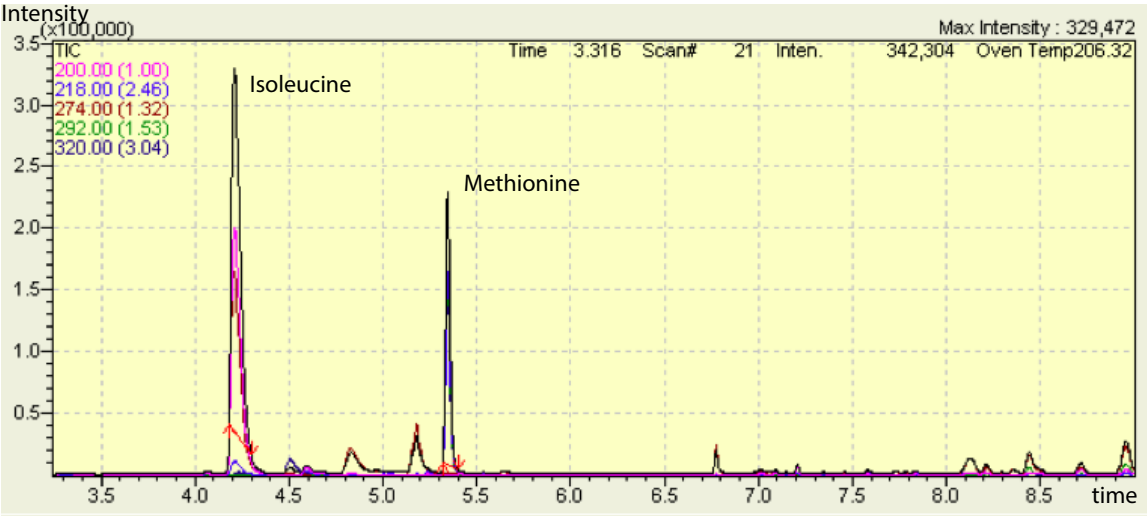
Supplemental Figure 1. Evolved cooperators with ancestral *metA* alleles fail to cooperate. Asterisks indicate presence of native allele (no substitution)



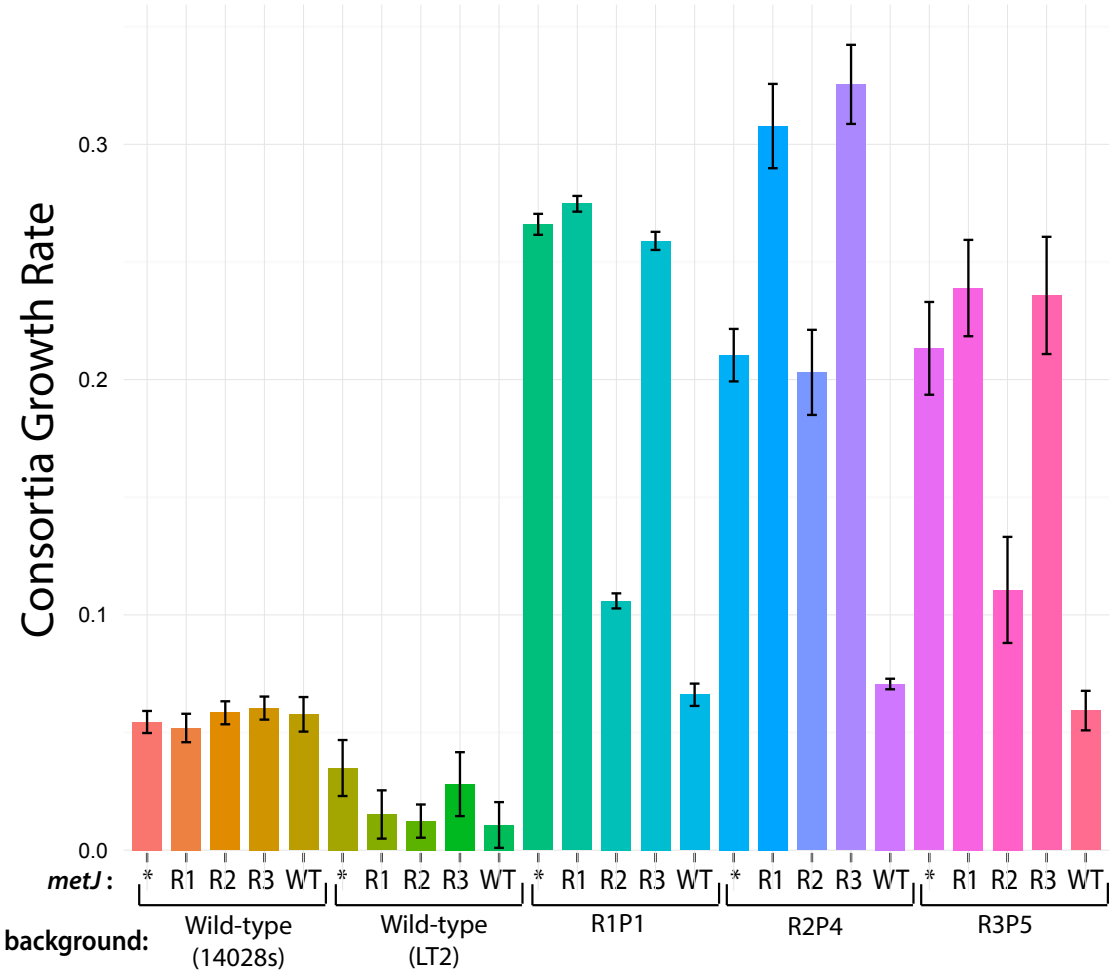
Supplemental Figure 2. Epitope tagging ancestral *metA* allele cconsortia growth in ancestral background. Each data point represents at least three technical replicates. Values of tags in ancestral backgrounds represent grand mean of three (a,b) or four (c) biological replicates, each with three technical replicates. Red bars highlight cooperation in ancestral backgrounds with tagged *metA*.



Supplemental Figure 3. Consortia composition equilibria correlate with methionine production. YFP-labeled *S.enterica* and CFP-*labeled E. coli* Δ*metB* grown in (a) solid and (b) liquid media were sampled at various time points, and consortia composition determined via flow-cytometery. Each data point represents three biological replicates, each with three technical replicates.



Supplemental Figure 4. Example of GC-chromatogram of derivatized amino acids from cooperator spent media. This sample is one biological replicate of R1P2, with the methionine and isoleucine peaks clearly visible. Fragment patterns at each peak match those of identified derivatized amino acids. Area under each peak corresponds to amino acid quantity.



Supplemental Figure 5. Consortia growth rates for wild-type, R, and RP strains with substituted *metJ* alleles. $metJ^{WT}$ in wild-type background acted as a control. Error bars represent standard error of three biological replicates.

Supplemental Tables

Supplemental Table 1. Primers used in gene disruptions, replacements, and epitope tagging in Chapter 2.

Primer Name	Sequence	Template	Function
ΔmetA::cat F	ggatgtctaaacgtttaaacgtatgtcgtgag gttatcag gtgtaggctggagctgcttc	pKD32[1]	*metA* deletion
ΔmetA::cat R	aggcacccgaaggTGCCTGATTC AACATGCTGAAACGCTT ctgtcaaacatgagaattaa	pKD32	*metA* deletion
SMD22	gatcttccgtcacaggtagg	Δ*metA::cat*	internal cat primer to check insertion
SMD8	CTCACCTTGAACTTGCAGA C	chromosomal *metA*	amplify *metA* F
SMD9	ATGCGAACGAAGGATCACT G	chromosomal *metA*	amplify *metA* R
SMD1	TACGCCATATGATCTGCGT CACATGAATCCAACCCTGG ATGACTACAAAGACCATGA CGG	pSUB11[2]	3xFLAG HTS
SMD4	CAACATGCTGAAACGCTTT AGGATCGTCAGAAGCAGA AGACA TAT GAA TAT CCT CCT TAG	pSUB11	3xFLAG HTS
SMD40	TTGGATCCCGAACTGGAAG AACTGGTGAG	chromosomal *metA*	pKNOCK-*metA* cloning
SMD41	TTGGATCCCTCACCAGTTCT TCCAGTTCG	chromosomal *metA*	pKNOCK-*metA* cloning
SMD44	ACGTGTTCCGCTTCCTTTAG	pKNOCK-*metA*	external pKNOCK R to check insertion
SMD50	TTGGTCTCA GAGATCAGTTTCTGTTC ATCCAGGGTTGGATTCATG TGACG	pKNOCK[3]	EI-PCR construction of MyC HTS R
SMD51	TTGGTCTCATCTCTGAAGA AGACCTGTAATCTTCTGCTT CTGACGATCCT	pKNOCK	EI-PCR construction of MyC HTS F
SMD52	TTGGTCTCAGGAACATCAT ACGGATAATCCAGGGTTGG ATTCATGTGACG	pKNOCK	EI-PCR construction of HTS HA tag R (C terminus)
SMD53	TTGGTCTCATTCCTGATTAT GCTAGCCTCTAATCTTCTGC TTCTGACGATCCT	pKNOCK	EI-PCR construction of HTS HA tag F (C terminus)
SMD54	TTGGTCTCAGGAACATCAT ACGGATACATAACCTGATA ACCTCACGAC	pKNOCK	EI-PCR construction of HTS HA tag R (N terminus)
SMD55	TTGGTCTCA TTCCTGATTATGCTAGCCTC CCGATTCGCGTGCTGGAC	pKNOCK	EI-PCR construction of HTS HA tag F (N terminus)

Supplemental Table 2. The sequencing results for genomes of a) R2P4 and b) R3P5.

Rows in gray denote polymorphisms present in sequenced *S. enterica* 14028s ancestor.

a) R2P4

Reference Position	Variation Type	Reference	Allele Variations	Frequencies	Counts	Coverage	Overlapping Annotations	Amino Acid Change
609837	SNP	A	1	T	100	8	Gene: fimH, CDS: fimH	Ile157Phe
1314150	SNP	A	C	100	6	6		
1314156	SNP	A	G	100	6	6		
1328473	SNP	T	A	100	10	10	Gene: STM14_1478, CDS: STM14_1478	Asn625Lys
1723496	SNP	T	C	100	11	11	Gene: STM14_1964, CDS: STM14_1964	Thr362Ala
1969451	SNP	A	C	100	3	3	Gene: STM14_2254	
1978109	SNP	G	A	100	3	3	Gene: STM14_2274, CDS: STM14_2274	Gly117Asp
1978116	SNP	T	C	100	6	6	Gene: STM14_2274, CDS: STM14_2274	
2391971	SNP	T	A	100	6	6	Gene: STM14_2767, CDS: STM14_2767	Ser47Cys
2391985	SNP	A	T	100	10	10	Gene: STM14_2767, CDS: STM14_2767	Phe42Tyr
2480438	SNP	A	G	100	37	37	Gene: nuoL, CDS: nuoL	Val206Ala
2539542	SNP	C	A	80	4	5	Gene: STM14_2926, CDS: STM14_2926	Val179Phe
2539546	SNP	C	A	80	4	5	Gene: STM14_2926, CDS: STM14_2926	
2826322	SNP	T	A	100	6	6	Gene: STM14_3224, CDS: STM14_3224	Tyr512Asn
2826326	SNP	T	A	100	7	7	Gene: STM14_3224, CDS: STM14_3224	Leu513Gln
2835849	SNP	A	T	100	24	24	Gene: nadB, CDS: nadB	Ile530Phe
2917346	SNP	A	C	100	8	8	Gene: STM14_3323, CDS: STM14_3323	Phe100Leu
3086372	SNP	C	T	100	21	21	Gene: rpoS, CDS: rpoS	Asp118Asn
3213731	SNP	A	C	100	5	5		
4316301	SNP	T	A	100	11	11	Gene: cytR, CDS: cytR	Glu219Val
4323087	SNP	C	T	100	17	17		
4415061	SNP	C	T	100	8	8	Gene: metA, CDS: metA	Arg228Cys
4503221	SNP	G	C	100	16	16	Gene: STM14_5121, CDS: STM14_5121	

b) R3P5

Reference Position	Variation Type	Reference	Variations	Frequencies	Counts	Coverage	Overlapping Annotations	Amino Acid Change
609837	SNP	A	T	100	126	126	Gene: fimH, CDS: fimH	Ile157Phe
1328473	SNP	T	A	100	82	82	Gene: STM14_1478, CDS: STM14_1478	Asn657Lys
1431340	SNP	G	C	100	9	9	Gene: btuD, CDS: btuD	Arg57Ser
1723496	SNP	T	C	100	132	132	Gene: STM14_1964, CDS: STM14_1964	Thr362Ala
1978109	SNP	G	A	98.1	52	53	Gene: STM14_2274, CDS: STM14_2274	Gly117Asp
1978116	SNP	T	C	98.5	65	66	Gene: STM14_2274, CDS: STM14_2274	
2391971	SNP	T	A	100	77	77	Gene: STM14_2767, CDS: STM14_2767	Ser47Cys
2391985	SNP	A	T	100	124	124	Gene: STM14_2767, CDS: STM14_2767	Phe42Tyr
2480438	SNP	A	G	100	81	81	Gene: nuoL, CDS: nuoL	Val206Ala
2826322	SNP	T	A	100	63	63	Gene: STM14_3224, CDS: STM14_3224	Tyr512Asn
2826326	SNP	T	A	100	74	74	Gene: STM14_3224, CDS: STM14_3224	Leu513Gln
2835849	SNP	A	T	100	124	124	Gene: nadB, CDS: nadB	Ile530Phe
2917346	SNP	A	C	99	99	100	Gene: STM14_3323; disrupted by stop codon	
2990333	Complex SNP	G	G/C	60.0/ 40.0	12/8	20		
3086372	SNP	C	T	100	120	120	Gene: rpoS, CDS: rpoS	Asp118Asn
3213731	SNP	A	C	99.2	129	130		
4316301	SNP	T	A	100	139	139	Gene: cytR, CDS: cytR	Glu219Val
4323003	SNP	G	A	100	139	139	Gene: metJ, CDS: metJ	Pro11Ser
4414564	SNP	C	T	100	136	136	Gene: metA, CDS: metA	Pro62Leu
4503221	SNP	G	C	100	14	14	Gene: STM14_5121, CDS: STM14_5121	

Supplemental Table 3. Values for quantitative RT-PCR analysis of *metA* and *metJ* expression in R and RP strains relative to wild-type.

Strain	*metJ* expression (mean)	*metJ* expression (SE)	*metA* expression (mean)	*metA* expression (SE)
R1	1.2614139	0.16196897	47.9835204	8.1671824
R2	0.6077543	0.05412021	4.4542027	0.519118
R3	1.0301748	0.01328234	2.5316303	0.8302519
R1P1	2.4377143	2.04962277	28.8944162	18.3627719
R2P4	0.5147121	0.09699444	0.8460209	0.1927046
R3P5	1.1790125	0.09644948	1.038024	0.4634716
R0P9	3.3370292	0.25254367	35.1798639	9.0180233

Supplemental Table 4. Primers used in gene disruptions, replacements, and epitope tagging in Chapter 3.

Primer Name	Sequence	Template	Function
SMD25	caggttcagacctcaatattaatgacgaagaggattaagt gtgtaggctggagctgcttc	pKD32[1]	*metJ* deletion F
SMD60	agcgcggccagaggcgttctgaccgcatgctttgctaTCA cctacctgtgacggaagatc	pKD32	*metJ* deletionR
SMD22	gatcttccgtcacaggtagg	Δ*metJ*::cat	internal cat primer to check insertion
SMD30	cgagggttattcgggaagatg	chromosomal *metJ*	amplify *metJ* R
SMD60.1	TAGGTACTGGAGAGATGGATTG	chromosomal *metJ*	amplify *metJ* F
SMD62	GTCGAATTCTTATGACTCCTCC	mRNA/cDNA	*gry*B F (qPCR)
SMD63	CGTCGATAGCGTTATCTACC	mRNA/cDNA	*gryB* R (qPCR)
SMD68	CGGTTTCCATTCCTCTGAAGGTG	mRNA/cDNA	*metJ* F (qPCR)
SMD69	CAGTATTCCCACGTTTCCGG	mRNA/cDNA	*metJ* R (qPCR)
SMD72	GCTGGGTCTGGTGGAGTTTAATG	mRNA/cDNA	*metA* F (qPCR)
SMD73	GGAAATCGGCATAGCGTGAG	mRNA/cDNA	*metA* R (qPCR)

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